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Hybridization, ancestral polymorphism, and cryptic species in Nothonotus darters (Teleostei: Percidae: Etheostomatinae)

Benjamin Paul Keck
University of Tennessee

To the Graduate Council:

I am submitting herewith a dissertation written by Benjamin Paul Keck entitled "Hybridization, ancestral polymorphism, and cryptic species in Nothonotus darters (Teleostei: Percidae: Etheostomatinae)." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology and Evolutionary Biology.

Thomas J. Near, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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____Thomas J. Near_____
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We have read this dissertation
and recommend its acceptance:

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Vice Provost and
Dean of the Graduate School

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HYBRIDIZATION, ANCESTRAL POLYMORPHISM, AND CRYPTIC SPECIES IN *NOTHONOTUS* DARTERS (TELEOSTEI: PERCIDAE: ETHEOSTOMATINAE)

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Benjamin Paul Keck
May 2009

To Julie for having faith in my resolve to complete this study and Elizabeth for reminding me of the exuberance of discovering the beasts around us.

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ABSTRACT

Darters (Teleostei: Percidae: Etheostomatinae) are a diverse group of charismatic fish endemic to North America and many of their characteristics combined with the fact that diversity within the clade is relatively well known makes them an attractive system for studying evolutionary patterns. I used molecular and morphological data to identify patterns of hybridization in darters, introgression in *Nothonotus* darters, and small geographic scales of diversification in two Cumberland River drainage *Nothonotus* species. I compiled records of hybrid Etheostomatinae museum specimens and found that over one quarter of darter species were involved in hybrid specimens, species most frequently involved had large range sizes, and involvement was negatively correlated with phylogenetic distance. I created a *Nothonotus* phylogeny based on mitochondrial and nuclear DNA sequence data and morphological data, and the observed relationships were largely consistent with previous hypotheses; however, better resolution and sampling in this phylogeny identified novel relationships and paraphyly. I expanded genetic sampling of *N. camurus*, *N. chlorobranchius*, and *N. rufilineatus*, the three *Nothonotus* most frequently involved in hybrid specimens, to search for introgression. I found extensive mitochondrial replacement in *N. rufilineatus*, with those in the upper Tennessee River drainage having mitochondrial haplotypes similar to haplotypes observed in *N. chlorobranchius* and those in the Cumberland River drainage having mitochondrial haplotypes similar to haplotypes observed in *N. camurus*. Additionally, *N. rufilineatus* has acted as a 'conduit species' in the upper Tennessee River drainage by transferring *N. chlorobranchius* like haplotypes into *N. camurus*. I also expanded genetic sampling of *N. microlepidus* and *N. sanguifluus*, two Cumberland River drainage *Nothonotus* whose sister relationship had not previously been hypothesized, to identify the number of lineages within the Cumberland River drainage. I preformed Discriminant Analysis using meristic characters based on the lineages indicated by the genetic analyses and found that there were four distinct lineages. By comparing the diversity in these two 'species' to diversity in another subclade of darters, barcheeks, I concluded that the isolating mechanisms in the Cumberland River drainage occur at small geographic scales, as found in the barcheeks, have been persistent through significant evolutionary time, and across multiple darter subclades.

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CHAPTER 1
Introduction and overview

North America contains the most diverse teleost fish fauna of temperate regions (Lundberg et al. 2000) and in over 220 years of ichthyological research on this continent (Hocutt and Wiley 1986) this diversity has been relatively well documented. Having a thorough understanding of these fishes is ecologically informative (Etnier 1997; Jelks et al. 2008; Sutherland et al. 2002), allows comparative evolutionary studies (Bolnick and Near 2005; Collar et al. 2005; Mendelson 2003b), and makes this fauna more accessible to the general public, *e.g.*, North American Native Fishes Association, encouraging better stewardship through books of regional fauna or specific fish clades (Etnier and Starnes 1993; Kuehne and Barbour 1983; Page 1983; Page and Burr 1991). However, continuing investigations and refinement of modern techniques, such as molecular systematics, are providing increasingly detailed information on the evolutionary histories of fishes and geographic scales of diversification.

Darters (Percidae: Etheostomatinae) are a particularly charismatic and notably diverse clade of fishes endemic to North America (Jordan and Evermann 1896; Kuehne and Barbour 1983; Page 1983). There are over 225 species of darters in five subclades *Ammocrypta*, *Crystallaria*, *Etheostoma*, *Nothonotus* and *Percina*. Darter diversity is highest in the Central Highlands (Hocutt and Wiley 1986; Page 1983) where there are often multiple species in sympatry. Darters partition habitat mostly by substrate type and flow regime, but these associations can change during breeding seasons (Greenberg 1991; Winn 1958). Darters are sexually dimorphic, often including dramatic color differences, and demonstrate three general breeding behaviors: 1) egg buriers, 2) egg attachers, and 3) egg guarders (Page 1985). The facts that darters are a diverse clade, their distributions are well known, and their general biology is understood make them an attractive group for studying evolutionary processes.

I focus on the darter subclade *Nothonotus*, Putnam 1863, because of two observations I made early in my ichthyological career, described below. *Nothonotus* is a diverse subclade with 20 recognized species that are distributed throughout much of the Central Highlands and previously glaciated north, with up to four species in sympatry in some Eastern Highland river systems, and demonstrate two of the three darter breeding behaviors. *Nothonotus* has a basal node minimal age of approximately 18 mya (Near and Keck 2005). *Nothonotus* as diagnosed (Bailey 1959; Bailey and Etnier 1988; Etnier and Williams 1989; Page 1981; Zorach 1972) are characterized by: body elongate, laterally compressed, with a deep caudal peduncle; anterior interradial membranes of the spinous dorsal darkly pigmented; snout conical or sharp to blunt; infraorbital and supratemporal canals complete; lateral line complete (except in *N. denoncourti* and *N. tippecanoe*); gill membranes narrowly or widely conjoined; strong sexual dichromatism; male egg-guarding (*N. maculatus* group) or eggs buried and left unguarded. Etnier and Williams (1989) give the character of dark pigmentation in the anterior interradial membranes of the spinous dorsal as a synapomorphy that unites *Nothonotus* and separates it from all other *Etheostoma*. *Nothonotus* species generally inhabit areas of swift current in small streams to large rivers with gravel, cobble, and small boulder substrates (Etnier and

Starnes 1993; Kuehne and Barbour 1983; Page 1983). *Nothonotus* are among some of the most brilliantly colored darters with deep greens of *N. chlorobranchius* and *N. microlepidus*, brick red of *N. rufilineatus*, neon orange of *N. tippecanoe*, and the distinctive crimson spotting on the sides of most *Nothonotus*.

Nothonotus was recently elevated to generic status (Near and Keck 2005) from its subgeneric status within *Etheostoma* (Bailey and Etnier 1988; Bailey and Gosline 1955; Bailey et al. 1954; Page 1981). The latter authors relegated *Nothonotus* to subgeneric status citing low resolution of relationships within Etheostomatinae using available characters as well as for nomenclatural stability. Bailey and Gosline (1955), Etnier and Starnes (1993), and Page (1981) predicted future research would result in better resolution and re-elevation of at least some of the subclades, but Page (1981) recommended the continued use of a broadly inclusive *Etheostoma* citing the inherent nomenclatural stability of more inclusive genera. Near and Keck (2005) elevated *Nothonotus* citing the repeated recovery of the group as monophyletic (Sloss et al. 2004; Song et al. 1998), but there were objections (Lang and Mayden 2007) and these are discussed in Chapter 3. I maintain that recognizing *Nothonotus* provides a more informative classification both evolutionarily and ecologically, and warrants any temporary nomenclatural instability.

During regular curatorial work in the University of Tennessee Research Collection of Fishes I noted that there were hybrid specimens resulting from crosses among *Nothonotus* species and from this observation developed a research program to answer the question 'Is there genetic introgression in *Nothonotus* and if so, is it consistent with patterns observed in other darter subclades?' The 2nd, 3rd, and 4th chapters of this dissertation describe the extent of hybridization and introgression in *Nothonotus* specifically, and general patterns across darter subclades.

In the second chapter I compiled museum records of Etheostomatinae hybrid specimens to identify patterns of hybridization in the group. There had not been a compilation of such records in over 30 years and I used this data to guide my genetic exploration of *Nothonotus* in chapters 3 and 4. Within *Nothonotus* three species, *N. camurus*, *N. chlorobranchius*, and *N. rufilineatus*, were frequently identified as a parental species in museum hybrid specimens and in order to identify possible introgression I first created a 'backbone phylogeny' (chapter 3) using genetic and morphological data and restricted sampling of the three focal species from areas of allopatry. The topology of the three focal species was consistent with previous hypotheses (Etnier and Williams 1989; Wood 1996). In chapter 4 I expanded the sampling of the three focal species to include specimens from their entire ranges and sequenced one mitochondrial gene (cyt*b*) and three nuclear genes (S7 intron, MLL exon, RAG1 exon) to search for any discordance among the gene trees.

Hybridization occurs across the Tree of Life and has the potential to change the evolutionary trajectory of lineages (Arnold 1992, 1997, 2006; Avise 2004; Coyne and Orr 2004; Dowling et al. 1997; Levin 1979; Stebbins 1959; West-Eberhard 2003). Hybridization can result in new evolutionary lineages through genetic remixing that may be phenotypically diagnosable (Dowling and Demarais 1993; Nolte et al. 2005) cryptic (Alvarez and Wendel 2006; Meyer et al. 2006), or constrained to delineated zones (Gee 2004; Harrison 1993; Szymura and Barton 1991). Additionally, hybridization can result

in gene trees that are not representative of the true evolutionary relationships of organisms and this cautions against relying on single lines of molecular evidence for phylogenetic reconstruction (Avise 2004; Bensch et al. 2006; Chan and Levin 2005; C. E. Edwards et al. 2006; Funk and Omland 2003; Hey et al. 2004; W.P. Maddison 1997; McCracken and Sorenson 2005; Melo-Ferreira et al. 2005; Moore 1995; Rognon and Guyomard 2003; Sota and Vogler 2001; Voros et al. 2006). By describing introgression in *Nothonotus* this system may be used to experimentally explore the genetic, behavioral, and environmental contributions to this natural process.

In a study of the timing of diversification in *Nothonotus* darters (Near and Keck 2005) I noted that there were polyphyletic species and novel interspecific relationships in the *N. maculatus* species group (*N. aquali*, *N. maculatus*, *N. microlepidus*, *N. sanguifluus*, *N. vulneratus*, *N. wapiti*). *Nothonotus sanguifluus* was paraphyletic with *N. microlepidus* and this close relationship was a novel result, as previous hypothesized sister species for these two Cumberland River drainage species were from outside the Cumberland River drainage. This relationship indicated one within drainage diversification event, but the polyphyly limited identification of distinct lineages. The number of lineages within the Cumberland River drainage is interesting because a recent study of barcheek darters (Hollingsworth and Near in press) very small geographic scales of diversification were identified, and determining the number of *Nothonotus* lineages would allow comparison. Based on the observed *N. sanguifluus – N. microlepidus* relationship and small geographic scales of diversification I asked the question 'How many lineages of *N. sanguifluus* and *N. microlepidus* are there in the Cumberland River drainage, and is there a similar pattern to that seen in the barcheeks?' and this makes up Chapter 4. However, North American fish phylogeography has been primarily based on the relationships between allopatrically distributed clades occurring in major centers of diversity and river systems (Hocutt and Wiley 1986; Mayden 1988; Near and Keck 2005; Near et al. 2001; Ray et al. 2006; Wiley and Mayden 1985) and repeated patterns of small geographic scales of diversification would require identifying isolating mechanisms other than major geological events.

CHAPTER 2

Patterns of natural hybridization in darters (Percidae: Etheostomatinae)[1]

ABSTRACT

Hybridization is an evolutionarily important process with varied outcomes that depend on interacting factors of time since common ancestry, behavioral differences, and environmental conditions. Hybridization is relatively common in teleost fishes and patterns from naturally occurring hybrids and experimental interspecific crosses provide insight into the evolution of reproductive barriers that lead to speciation. It has been several decades since records of hybrid darter specimens (Percidae: Etheostomatinae) have been collected and analyzed. We assembled a dataset of 245 records from museum collections and literature representing 65 unique hybrid combinations involving 63 species. Frequencies of unique hybrid combinations decrease with phylogenetic distance and are lower between species with different egg deposition behaviors. This dataset likely underestimates the amount of hybridization that has occurred among darter species, because of the relatively narrow evolutionary time frame during which specimens have been collected and identified.

INTRODUCTION

The nearly ubiquitous use of molecular methods in organismal biology, *e.g.*, molecular phylogenetics, has revealed that hybridization and introgression are processes that are a part of the evolutionary legacy of clades across the Tree of Life (Arnold 2006). Genetic exchange between species can variably influence evolutionary trajectories and diversification (Anderson and Stebbins 1954; Arnold 1997, 2006; Barton 2001; Coyne and Orr 2004; Dowling and Secor 1997; P. R. Grant et al. 2005; V. Grant 1981; Levin 1979; Seehausen 2004; Stebbins 1959; West-Eberhard 2003) including the creation of novel lineages (Dowling and Demarais 1993; Durand et al. 2000; Gerber et al. 2001; Meyer et al. 2006; Nolte et al. 2005; Salzburger et al. 2002) or the extinction of lineages (Epifanio and Philipp 2000; Konishi and Takata 2004; Rhymer and Simberloff 1996; Wolf et al. 2001). The variable outcomes of hybridization depend on the interaction of several intrinsic and extrinsic factors including environmental variables, behavioral differences, phylogenetic relationships, and time to common ancestry. A general pattern of hybridization is when a change in environment disrupts prezygotic reproductive isolating barriers (RIB) and time to common ancestry determines genetic compatibility (Bolnick and Near 2005; Coyne and Orr 1989; Seehausen et al. 1997).

Prezygotic RIBs are usually the first barriers to evolve between closely related species and may be the first to breakdown(Coyne and Orr 2004), usually in disturbed or novel environments (Anderson 1948; Harrison 1993; C.L. Hubbs 1955). Disturbed, novel and homogenized habitats can block or interrupt signals associated with mate choice

[1] A slightly modified version of this chapter is in review as: Keck, B.P. and T.J. Near. Patterns of hybridization in darters (Percidae: Etheostomatinae). *American Midland Naturalist*. My contributions to this manuscript include: 1) development of ideas, 2) data collection and analyses, 3) most of the writing.

(Seehausen et al. 1997), reduce spawning site segregation (Taylor et al. 2006) and disrupt ecological isolation (Funk et al. 2006; Seehausen et al. 2008). Greater disparity in signals and complex behavior require greater perturbations to mask signals and do so at each step in the behavior. It is expected that hybridization will be more common between species with similar and simple mating modes and in altered environments.

Among vertebrates, hybridization is particularly common in freshwater teleost fishes and interest in patterns of hybridization of teleosts has been increasing over the past 50 years (Avise 2004; Bolnick and Near 2005; Campton 1987; C. Hubbs and Strawn 1957b; C.L. Hubbs 1955; Mendelson 2003a, b; Schwartz 1972; Scribner et al. 2001). Identification of hybrid teleost fishes is usually accomplished through comparisons of external morphological characters of the hybrid individual and hypothesized parental species (C.L. Hubbs 1955; Jenkins and Burkhead 1994; Scribner et al. 2001). Hybrid specimens are often intermediate in meristic and morphometric characters used to differentiate and diagnose the parental species (Hubbs and Strawn, 1957b; Neff and Smith, 1979; Ross and Cavender, 1981; Jenkins and Burkhead, 1994). However, hybrid intermediacy is variable and using morphology to differentiate between first generation, subsequent generation or back cross individuals may be misleading (C. Hubbs and Strawn 1957a; Ross and Cavender 1981). Molecular markers from the mitochondrial and nuclear genomes can confirm parental species (Ray et al. 2008), and have identified hybrids not revealed by morphological examinations (Page et al. 1992; Piller et al. 2008; Scribner et al. 2001).

Darters (Percidae: Ethestomatinae) are one of the most diverse clades of endemic North American teleost fishes with approximately 225 species classified in five major subclades (*Ammocrypta*, *Crystallaria*, *Etheostoma*, *Nothonotus* and *Percina*). Species diversity of darters is concentrated in the Interior Highlands and Eastern Highlands (Hocutt and Wiley 1986; Page 1983) and darter communities often contain multiple species that occur in sympatry. Darters partition habitat mostly by substrate type and flow regime, but these associations can change during breeding seasons (Greenberg 1991; Winn 1958). Many darter species are sexually dimorphic, including dramatic color differences, and demonstrate three general egg deposition behaviors: i) egg buriers bury clumps of eggs in loose substrate with no subsequent parental care, ii) egg attachers attach single eggs to various substrates with no subsequent parental care, and iii) the most complex behavior involves males defending a crevice under a suitable structure where females enter and deposit eggs and the male tends the fertilized eggs until hatching (Page 1985).

The first analyses of naturally occurring darter hybrids and experimental crosses indicated reduced hybridization between more distantly related species (C. Hubbs and Strawn 1957b; C.L. Hubbs 1955). Most of the literature on darter hybrids subsequent to C. L. Hubbs (1955) focused on reporting and documenting individual occurrences of hybrid specimens (Howell and Boschung 1966; Mayden and Burr 1980; Page 1976). More recently, patterns and causes of hybridization have been investigated using allozyme data and phylogenetic analysis of DNA sequence data and have identified instances of introgression from complete mitochondrial replacement to geographically limited genetic exchange (Eisenhour 1995; F. D. Martin and Richmond 1973; Ray et al. 2008). Observation followed by hypothesis testing is the natural progression of science.

However, new observations need incorporation into the dialogue and it has been several decades since occurrences of natural hybrid teleost fishes have been thoroughly reviewed (C.L. Hubbs 1955; Schwartz 1972).

During routine curatorial practices at the University of Tennessee Research Collection of Fishes (UT) we noted the presence of hybrid darter specimens resulting from interspecific crosses that were previously unreported. A subsequent search of other fish collections supported our suspicion that there were many additional hybrid darter specimens resulting from interspecific crosses that have not been previously reported. We decided to expand the search for darter hybrid specimens and investigate correlations of darter hybridization with phylogenetic relationships and reproductive behaviors of species involved with hybridization.

METHODS

We obtained records of putative hybrid darter specimens through a literature review (Branson and Campbell 1969; Echelle et al. 1974; Eisenhour 1995; Etnier and Bailey 1989; Hocutt and Hambrick 1973; Howell and Boschung 1966; C Hubbs et al. 1988; 1961a; C Hubbs and Laritz 1961b; C. Hubbs and Strawn 1957a; 1957b; Linder 1955; Loos and Woolcott 1969; Mayden and Page 1979; Page 1974a; 1976; Page et al. 1992; Schwartz 1972; Suttkus and Ramsey 1967) and by contacting curators or searching online databases at the following institutions: AMNH, ANSP, AUM, CAS, CU, EUK, FMNH, INHS, JFBM, KU, MMNS, Morehead State University, NCSM, OKMNH, OSUM, RC, ROM, SIUC, TNHC, TU, UAIC, UMMZ, UT, and YPM; institutional abbreviations are as listed in Leviton *et al.* (1985) and Leviton (1988) except for The Ohio State University (OSUM and not OSU), or the full name of the institution is listed here. Experimental hybrids were not included in our study. Hybrid specimens within the *E. blennioides* species complex, *E. nigrum-E. olmstedi* species complex and *Percina caprodes* species complex were excluded because of unresolved taxonomic and phylogenetic issues within these clades (Chapleau and Cooper 1992; Chapleau and Pageau 1985; Kuehne and Barbour 1983; Near 2008; Page 1983; Piller et al. 2008). To avoid over estimating the number of hybrid crosses we did not include reported intergrades between taxonomically defined subspecies. Hybrid specimens are listed alphabetically and parental species order does not imply the sex of parental species in the cross.

RESULTS

Our review of the literature and collection records resulted in 245 hybrid accounts (Table A.1). We found 65 unique crosses involving 63 species distributed among four of the five major clades (*Ammocrypta*, *Etheostoma*, *Nothonotus* and *Percina*), with no hybrids involving either of the two *Crystallaria* species (Table 2.1). Species were represented in records between 1 and 71 times, with 41.3% of species represented in only one record and 17.5% represented in more than 10 records (Fig. 2.1). Among the unique crosses, there were 41 that are represented by a single record and four crosses were represented by more than 10 records (Fig. 2.1). Hybrids between closely related species in the *E. squamiceps* clade comprised 21 of the records. Over one-third of the records represent specimens taken from the same locality at different collection dates. The

Table 2.1. Percentage involvement of species and occurrence of crosses. Clades and their respective species are listed alphabetically in the columns 'Clade' and 'Species'. Every species involved is listed under 'Species' resulting in reciprocal crosses being listed. Numbers and percentages given under 'Involvement' are as follows: a) for a clade – number of records involving that clade : number of unique crosses in that clade (percentage of all records involving that clade : percentage of all unique crosses found in that clade) b) for a species – number of records involving that species (percentage of all records involving that species : percentage of all clade records involving that species). Under 'Cross' to the right of each species or clade listed under 'Species' or 'Clade', respectively, is a list of all species or clades it is involved in a cross with. Numbers and percentages under 'Occurrence' are as follows: a) for a clade – number of records involving those two clades (percentage of all records involving those two clades : percentage of all clade records involving that cross : percentage of all interclade crosses involving those two clades) b) for a species – number of records involving those specific species (percentage of all records involving those two species : percentage of all clade records involving those two species)

Clade	Species	Invovlement	Cross	Occurrence
Ammocrypta		2:1 (0.8%:1.5%)		
	A. beanii	2 (0.8%:100%)	X *A. meridiana*	2 (0.8%:100%)
	A. meridiana	2 (0.8%:100%)	X *A. beanii*	2 (0.8%:100%)
Etheostoma		76:30 (31%:46.1%)	X *Nothonotus*	1 (0.4%:1.3%:7.7%)
			X *Percina*	8 (3.3%:10.5%:61.5%)
	E. artesiae	1 (0.4%:1.3%)	X *E. stigmaeum*	1 (0.4%:1.3%)
	E. blennioides	2 (0.8%:2.6%)	X *E. caeruleum*	1 (0.4%:1.3%)
			X *E. spectabile*	1 (0.4%:1.3%)
	E. caeruleum	28 (11.4%:36.8%)	X *E. blennioides*	1 (0.4%:1.3%)
			X *E. fragi*	1 (0.4%:1.3%)
			X *E. lawrencei*	2 (0.8%:2.6%)
			X *E. spectabile*	21 (8.6%:27.6%)
			X *N. rufilineatus*	1 (0.4%:1.3%)
			X *P. caprodes*	1 (0.4%:1.3%)
			X *P. maculata*	1 (0.4%:1.3%)
	E. chlorosoma	2 (0.8%:2.6%)	X *E. gracile*	1 (0.4%:1.3%)
			X *E. stigmaeum*	1 (0.4%:1.3%)
	E. collis	2 (0.8%:2.6%)	X *E. olmstedi*	2 (0.8%:2.6%)
	E. corona	5 (2%:6.6%)	X *E. nigripinne*	5 (2%:6.6%)
	E. crossopterum	8 (3.3%:10.5%)	X *E. nigripinne*	8 (3.3%:10.5%)
	E. duryi	1 (0.4%:1.3%)	X *E. simoterum*	1 (0.4%:1.3%)
	E. flavum	1 (0.4%:1.3%)	X *E. planasaxatile*	1 (0.4%:1.3%)
	E. forbesi	8 (3.3%:10.5%)	X *E. nigripinne*	8 (3.3%:10.5%)
	E. fragi	1 (0.4%:1.3%)	X *E. caeruleum*	1 (0.4%:1.3%)
	E. gracile	2 (0.8%:2.6%)	X *E. chlorosoma*	1 (0.4%:1.3%)
			X *P. maculata*	1 (0.4%:1.3%)
	E. kennicotti	1 (0.4%:1.3%)	X *E. squamiceps*	1 (0.4%:1.3%)
	E. lawrencei	2 (0.8%:2.6%)	X *E. caeruleum*	2 (0.8%:2.6%)
	E. lepidum	2 (0.8%:2.6%)	X *E. spectabile*	1 (0.4%:1.3%)
			X *P. caprodes*	1 (0.4%:1.3%)
	E. nigripinne	21 (8.6%:27.6%)	X *E. corona*	5 (2%:6.6%)
			X *E. crossopterum*	8 (3.3%:10.5%)
			X *E. forbesi*	8 (3.3%:10.5%)
	E. olmstedi	2 (0.8%:2.6%)	X *E. collis*	2 (0.8%:2.6%)
	E. planasaxatile	1 (0.4%:1.3%)	X *E. flavum*	1 (0.4%:1.3%)
	E. punctulatum	1 (0.4%:1.3%)	X *E. spectabile*	1 (0.4%:1.3%)
	E. radiosum	7 (2.9%:9.2%)	X *E. spectabile*	7 (2.9%:9.2%)

Table 2.1. Continued.

Clade	Species	Invovlement	Cross	Occurrence
	E. ramseyi	1 (0.4%:1.3%)	X *E. rupestre*	1 (0.4%:1.3%)
	E. raneyi	1 (0.4%:1.3%)	X *P. nigrofasciata*	1 (0.4%:1.3%)
	E. rupestre	1 (0.4%:1.3%)	X *E. ramseyi*	1 (0.4%:1.3%)
	E. simoterum	1 (0.4%:1.3%)	X *E. duryi*	1 (0.4%:1.3%)
	E. spectabile	34 (13.9%:44.7%)	X *E. blennioides*	1 (0.4%:1.3%)
			X *E. caeruleum*	21 (8.6%:27.6%)
			X *E. lepidum*	1 (0.4%:1.3%)
			X *E. punctulatum*	1 (0.4%:1.3%)
			X *E. radiosum*	7 (2.9%:9.2%)
			X *E. whipplei*	1 (0.4%:1.3%)
			X *P. caprodes*	1 (0.4%:1.3%)
			X *P. sciera*	1 (0.4%:1.3%)
	E. squamiceps	1 (0.4%:1.3%)	X *E. kennicotti*	1 (0.4%:1.3%)
	E. stigmaeum	3 (1.2%:3.9%)	X *E. artesiae*	1 (0.4%:1.3%)
			X *E. chlorosoma*	1 (0.4%:1.3%)
			X *E. tallapoosae*	1 (0.4%:1.3%)
	E. tallapoosae	1 (0.4%:1.3%)	X *E. stigmaeum*	1 (0.4%:1.3%)
	E. whipplei	1 (0.4%:1.3%)	X *E. spectabile*	1 (0.4%:1.3%)
	E. zonale	1 (0.4%:1.3%)	X *P. caprodes*	1 (0.4%:1.3%)
Nothonotus		34:12 (13.9%:18.5%)	X *Etheostoma*	1 (0.4%:2.9%:7.7%)
			X *Percina*	4 (1.6%:11.8%:30.8%)
	N. acuticeps	1 (0.4%:2.9%)	X *N. chlorobranchius*	1 (0.4%:2.9%)
	N. camurus	14 (5.7%:41.2%)	X *N. chlorobranchius*	1 (0.4%:2.9%)
			X *N. microlepidus*	1 (0.4%:2.9%)
			X *N. rufilineatus*	9 (3.7%:26.5%)
			X *N. tippecanoe*	3 (1.2%:8.8%)
	N. chlorobranchius	13 (5.3%:38.2%)	X *N. acuticeps*	1 (0.4%:2.9%)
			X *N. camurus*	1 (0.4%:2.9%)
			X *N. rufilineatus*	11 (4.5%:32.3%)
	N. microlepidus	2 (0.8%:5.9%)	X *N. camurus*	1 (0.4%:2.9%)
			X *N. rufilineatus*	1 (0.4%:2.9%)
	N. rufilineatus	28 (11.4%:82.5%)	X *N. camurus*	9 (3.7%:26.5%)
			X *N. chlorobranchius*	11 (4.5%:32.3%)
			X *N. microlepidus*	1 (0.4%:2.9%)
			X *N. vulneratus*	2 (0.8%:5.9%)
			X *E. caeruleum*	1 (0.4%:2.9%)
			X *P. caprodes*	1 (0.4%:2.9%)
			X *P. evides*	1 (0.4%:2.9%)
			X *P. squamata*	2 (0.8%:5.9%)
	N. tippecanoe	3 (1.2%:8.8%)	X *N. camurus*	3 (1.2%:8.8%)
	N. vulneratus	2 (0.8%:5.9%)	X *N. rufilineatus*	2 (0.8%:5.9%)
Percina		146:34 (59.6%:52.3%)	X *Etheostoma*	8 (3.3%:5.5%:61.5%)
			X *Nothonotus*	4 (1.6%:2.7%:30.7%
	P. aurantiaca	1 (0.4%:0.7%)	X *P. caprodes*	1 (0.4%:0.7%)
	P. aurolineata	1 (0.4%:0.7%)	X *P. lenticula*	1 (0.4%:0.7%)
	P. burtoni	1 (0.4%:0.7%)	X *P. sciera*	1 (0.4%:0.7%)
	P. caprodes	59 (24.1%:40.4%)	X *P. aurantiaca*	1 (0.4%:0.7%)
			X *P. copelandi*	3 (1.2%:2%)
			X *P. evides*	1 (0.4%:0.7%)

Table 2.1. Continued.

Clade	Species	Invovlement	Cross	Occurrence
			X *P. maculata*	28 (11.4%:19.2%)
			X *P. phoxocephala*	6 (2.4%:4.1%)
			X *P. sciera*	3 (1.2%:2%)
			X *P. shumardi*	6 (2.4%:4.1%)
			X *P. williamsi*	4 (1.6%:2.7%)
			X *E. caeruleum*	1 (0.4%:0.7%)
			X *E. lepidum*	1 (0.4%:0.7%)
			X *E. spectabile*	1 (0.4%:0.7%)
			X *E. zonale*	1 (0.4%:0.7%)
			X *N. rufilineatus*	1 (0.4%:0.7%)
	P. copelandi	4 (1.6%:2.7%)	X *P. caprodes*	3 (1.2%:2%)
			X *P. maculata*	1 (0.4%:0.7%)
	P. evides	2 (0.8%:1.4%)	X *P. caprodes*	1 (0.4%:0.7%)
			X *N. rufilineatus*	1 (0.4%:0.7%)
	P. kathae	1 (0.4%:0.7%)	X *P. smithvanizi*	1 (0.4%:0.7%)
	P. lenticula	1 (0.4%:0.7%)	X *P. aurolineata*	1 (0.4%:0.7%)
	P. macrocephala	2 (0.8%:1.4%)	X *P. caprodes*	2 (0.8%:1.4%)
	P. maculata	33 (13.5%:22.6%)	X *P. caprodes*	28 (11.4%:19.2%)
			X *P. copelandi*	1 (0.4%:0.7%)
			X *P. phoxocephala*	2 (0.8%:1.4%)
			X *E. caeruleum*	1 (0.4%:0.7%)
			X *E. gracile*	1 (0.4%:0.7%)
	P. nigrofasciata	65 (26.5%:44.5%)	X *P. sciera*	63 (25.7%:43.1%)
			X *P. shumardi*	1 (0.4%:0.7%)
			X *E. raneyi*	1 (0.4%:0.7%)
	P. notogramma	3 (1.2%:2%)	X *P. peltata*	3 (1.2%:2%)
	P. oxyrhyncha	1 (0.4%:0.7%)	X *P. roanoka*	1 (0.4%:0.7%)
	P. palmaris	1 (0.4%:0.7%)	X *P. smithvanizi*	1 (0.4%:0.7%)
	P. peltata	3 (1.2%:2%)	X *P. notogramma*	3 (1.2%:2%)
	P. phoxocephala	11 (4.5%:7.5%)	X *P. caprodes*	6 (2.4%:4.1%)
			X *P. maculata*	2 (0.8%:1.4%)
			X *P. sciera*	2 (0.8%:1.4%)
			X *P. shumardi*	1 (0.4%:0.7%)
	P. roanoka	1 (0.4%:0.7%)	X *P. oxyrhyncha*	1 (0.4%:0.7%)
	P. sciera	71 (29%:48.6%)	X *P. burtoni*	1 (0.4%:0.7%)
			X *P. caprodes*	3 (1.2%:2%)
			X *P. nigrofasciata*	63 (25.7%:43.1%)
			X *P. phoxocephala*	2 (0.8%:1.4%)
			X *P. sipsi*	1 (0.4%:0.7%)
			X *E. spectabile*	1 (0.4%:0.7%)
	P. shumardi	9 (3.7%:6.2%)	X *P. caprodes*	6 (2.4%:4.1%)
			X *P. nigrofasciata*	1 (0.4%:0.7%)
			X *P. phoxocephala*	1 (0.4%:0.7%)
			X *P. vigil*	1 (0.4%:0.7%)
	P. sipsi	1 (0.4%:0.7%)	X *P. sciera*	1 (0.4%:0.7%)
	P. smithvanizi	2 (0.8%:1.4%)	X *P. kathae*	1 (0.4%:0.7%)
			X *P. palmaris*	1 (0.4%:0.7%)
	P. squamata	2 (0.8%:1.4%)	X *N. rufilineatus*	2 (0.8%:1.4%)
	P. vigil	1 (0.4%:0.7%)	X *P. shumardi*	1 (0.4%:0.7%)
	P. williamsi	4 (1.6%:2.7%)	X *P. caprodes*	4 (1.6%:2.7%)

majority of these were *P. nigrofasciata* X *P. sciera* hybrids from a set of localities on the Pearl River (Suttkus and Ramsey 1967).

Distributing the unique hybrid crosses into categories of parental species egg deposition behavior revealed that most hybrid crosses involve egg buriers and all hybrid crosses of parental species that exhibit different egg deposition behaviors always involved egg buriers (Fig. 2.2). All but one hybrid cross involving egg attachers were represented by only one record and hybrid crosses involving egg guarders or egg buriers were often represented by more than one record. *Nothonotus microlepidus* and *N. vulneratus* are members of the *N. maculatus* clade (Keck and Near 2008; Near and Keck 2005), a monophyletic group of egg guarders, and were both involved in crosses with *N. rufilineatus*, an egg burier that is also the most common *Nothonotus* species involved in hybrid crosses. The remaining hybrid crosses involving egg guarding species were restricted to the *E. squamiceps* clade.

Clade and species involvement in hybrid crosses and frequency of crosses are listed in Table 2.1. The most unique hybrid crosses involved *Percina*, followed by *Etheostoma*, *Nothonotus*, and *Ammocrypta*. The most common hybrid crosses in *Percina* were *P. nigrofasciata* X *P. sciera* (44.5% of *Percina* hybrid accounts), and *Percina caprodes* X *P. maculata* (19.2% of *Percina* hybrid crosses). *Percina sciera*, *P. nigrofasciata*, *P. caprodes* and *P. maculata* were the species most frequently involved with other hybrid crosses. The only hybrid cross involving *Etheostoma* species represented by more than 10 records was *E. caeruleum* X *E. spectabile*. *Etheostoma spectabile* was involved in the most *Etheostoma* crosses followed by *E. caeruleum* and *E. nigripinne* with 36.8% and 27.6%, respectively, of the hybrid crosses involving *Etheostoma* species. All hybrid crosses involving *Nothonotus* species included at least one of the following three species, listed in decreasing frequency, *N. rufilineatus*, *N. camurus* and *N. chlorobranchius*. There was only one unique hybrid cross involving *Ammocrypta* species and this was represented in two records. All but one of the interclade hybrid crosses involved *Percina*, none involve *Ammocrypta*, and only one of these interclade hybrid crosses, *N. rufilineatus* X *P. squamata*, was represented in multiple records.

DISCUSSION

We are assuming that most instances of hybridization between darter species are not the result of chance fertilization through the accidental meeting of gametes in close proximity. The potential for random meetings is greatly restricted by the short average sperm motility time for many fish species (Alavi and Cosson 2005; 2006). Previous reviews of hybridization in freshwater actinopterygian fishes reported both far fewer darter hybrid crosses and involved fewer species than found in our analyses (C.L. Hubbs 1955; Scribner et al. 2001). The increase in reported hybrid darter crosses presented in our study is a result of greater access to museum collections and the accumulation of a greater number of specimens in research museum collections. Given this new review of darter hybrid crosses, we can interpret observed patterns and make predictions regarding genetic exchange between darter species.

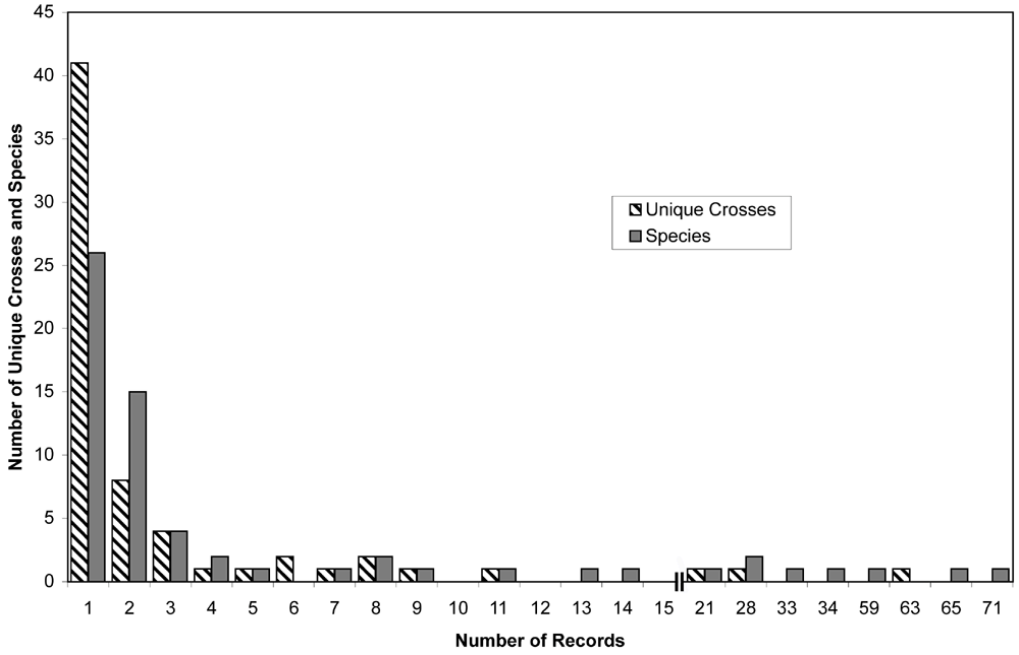

Fig. 2.1. Number of unique crosses and species by the number of records representing them.

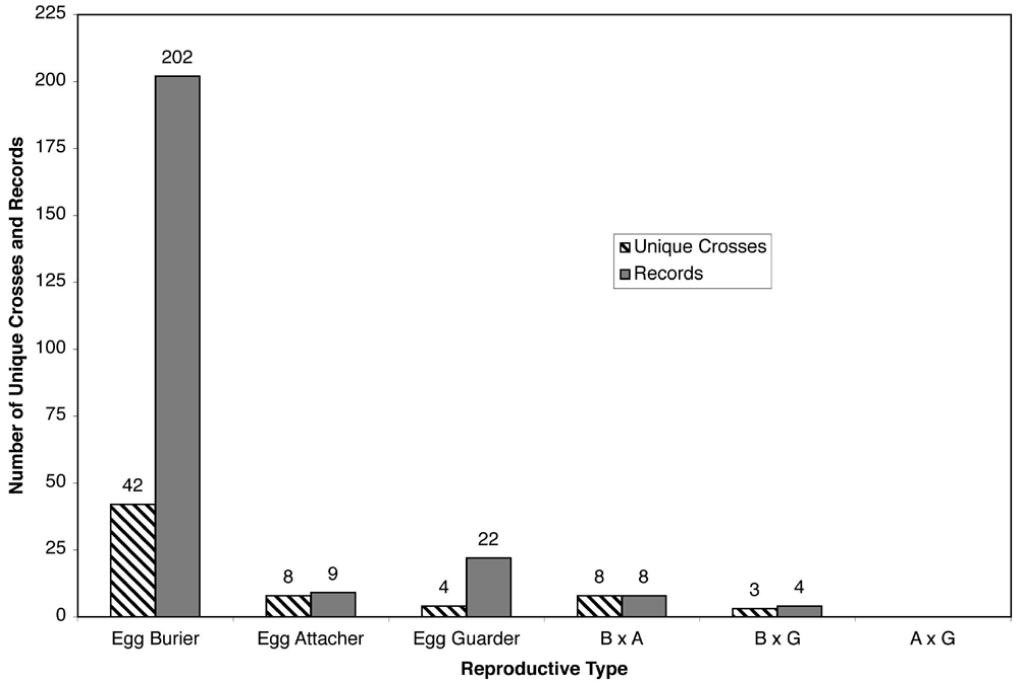

Fig. 2.2. Number of unique crosses and number of records by the breeding behaviors of the parental species. If both parental species are Egg Buriers then that cross and all records representing that cross were counted for that category. The other categories are 'Egg Attachers', 'Egg Gaurders', 'B x A' = one parent an Egg Burier and one an Egg Attacher, 'B x G' = one parent an Egg Burier and one an Egg Guarder, and 'A x G' = one parent an Egg Attacher and one an Egg Guarder.

Comparing the frequencies of hybrid crosses observed between darter subclades and egg deposition behaviors identified characteristics commonly associated with hybridization. Instances of hybridization between species generally decrease with increase in the time since common ancestry (Bolnick and Near 2005; Coyne and Orr 1989) and teleost fishes followed this pattern (Bolnick and Near 2005; Bolnick et al. 2008; Russell 2003; Scribner et al. 2001). The majority of observed hybrid crosses were between species within the same clade (*Ammocrypta*, *Etheostoma*, *Nothonotus* and *Percina*) and only 5.3% of the observed hybrid crosses involved species from different clades. Experimental crosses between darter species have been used to infer phylogenetic relationships (C. Hubbs and Strawn 1957b) and investigate patterns of reproductive isolation (Mendelson 2003a; 2003b; Mendelson et al. 2007). However, naturally occurring hybrid crosses are conditional on more factors than just time since common ancestry, including factors usually not considered in experimental crosses such as whether the parental species occur in sympatry, exhibit similar habitat preferences, or share similar annual spawning seasons. For example, there were more *Percina* X *Etheostoma* and *Percina* X *Nothonotus* hybrid crosses than *Etheostoma* X *Nothonotus* hybrid crosses, but this pattern does not necessarily reflect the phylogenetic relationships among these clades. Phylogenies inferred from mitochondrial genes indicated that *Nothonotus* was more closely related to *Percina* than *Etheostoma* (Lang and Mayden 2007; Sloss et al. 2004; Song et al. 1998), and those inferred from nuclear genes indicated that *Nothonotus* was more closely related to *Etheostoma* than *Percina* (Lang and Mayden 2007). If the pattern of natural crosses were to follow only phylogenetic relationship we would expect to see more *Percina* X *Nothonotus* and *Nothonotus* X *Etheostoma* hybrids than *Percina* X *Etheostoma*, but there was only one *Nothonotus* X *Etheostoma* hybrid and more *Percina* X *Etheostoma* hybrids than *Percina* X *Nothonotus* hybrids.

Isolating barriers are frequently studied in the context of hybridization (Bolnick and Near 2005; Brown et al. 2004; Lessios 2007; Linn et al. 2004; N. H. Martin and Willis 2007; Mendelson et al. 2007; Nagata et al. 2007; Vigueira et al. 2008; Whiteman and Gage 2007) and this dataset provides insight into the contributions of prezygotic isolating barriers in darters. Scribner *et al.* (2001) noted that there are fewer instances of hybridization as prezygotic behavior becomes more complex in teleost fishes. The egg deposition behaviors of darters can be categorized into egg buriers, egg attachers and egg guarders (Page 1985). The frequency of darter hybrid crosses does decrease with complexity of egg deposition behavior, with the majority of hybrid crosses involving egg burying species (Fig. 2.2), followed by hybrid crosses between species that attach eggs, and only four hybrid crosses between egg guarding species. Crosses involving species exhibiting the most complex egg deposition behavior are closely related and may reflect a lack of postzygotic barriers among these closely related species. This disproportionate distribution may be an artifact of species abundance and patterns of species sympatry, because egg guarding species are usually not sympatric with each other and there are few egg guarding species compared to many species of egg buriers that often occur in sympatry. However, egg guarders are usually sympatric with multiple egg buriers and if frequency of sympatry is the sole predictor of hybrid occurrence there should be more egg guarder by egg burier crosses than within egg guarder crosses, but the opposite pattern is observed (Fig. 2.2). Fewer between egg deposition type crosses are expected,

because isolation should be more effective between species exhibiting different egg deposition modes given fewer shared behaviors between the parental species. Many egg buriers look for similar substrates resulting in a higher chance that spawning individuals of different species will be in close proximity, thus increasing the likelihood of hybridization. The high number of records per unique cross in the egg buriers and egg guarders is related to the egg deposition behaviors, because both involve the deposition and fertilization of multiple eggs in one spawning act. In contrast, egg attachers deposit and fertilize only one egg per spawning act. Given equal viability of hybrids, offspring resulting from egg attacher hybrid crosses would be less abundant than those from egg guarders or attachers.

Anthropogenic alterations or disturbance of habitat are often cited as causes for the breakdown of RIBs (Scribner et al. 2001) and similar disturbances are threatening many aquatic systems (Etnier 1997; Lydeard and Mayden 1995). Assuming anthropogenic disturbances have been increasing over time, hybridization may be increasing as well. Many of the single instances of unique hybrid crosses and species involvement were from fairly recent field collections, possibly indicating a recent trend towards a higher frequency of breakdown among RIBs. However, the increasing numbers of identified hybrid crosses may also be correlated with increasing awareness of hybrid specimens by researchers, or more researchers and increased sampling effort.

Even if darter species exhibit female mate choice there is potential for males to make incorrect mate choices during cuckoldry. The dominant hybrid crosses in *Nothonotus* were between species in which the males are noticeably different in coloration, but the females are similar and all are egg buriers that select very similar spawning sites (Etnier and Starnes 1993). Cuckoldry occurs in *Nothonotus* (Fisher 1990), and Stiles (1988) suggested that young males of *N. rufilineatus* exhibit sneaking behavior. Similarity between the females may reduce a sneaking male's abilities to make correct mate choices. If one species were more likely to engage in cuckoldry a genetic analysis may reveal an asymmetrical introgression of the mitochondria genome, as observed in *E. uniporum* (Ray et al. 2008).

The species with the highest frequency of involvement in hybrid crosses exhibit relatively large geographic ranges that extend into or include the more species rich regions of North America (Hocutt and Wiley 1986; Page 1983), and are fairly abundant where they occur. Large ranges, sympatry with more species, and high abundance increases the potential for more unique hybrid crosses, but there are darter species with larger ranges that are rarely involved in hybridization. One of the most frequent hybrid crosses was between *P. caprodes* and *P. maculata*, two species that are not similar in appearance and not closely related relative to other *Percina* species (Near 2002), but do have broadly overlapping ranges, are abundant where they occur, and are both egg buriers. Large range size is not the agent of hybridization, but the characteristics that enable large range size, or increased exposure to novel and disturbed environments confer the potential for hybridization.

With more than 25% of all darter species involved with hybrid crosses perhaps the most pertinent observation is that hybridization is not an anomaly in the clade, and there is ample opportunity for genetic exchange between species. We can make predictions regarding the attributes a darter species is likely to have if it is frequently

involved in hybrid crosses. These species are usually in the same major darter clade, exhibit the same egg deposition behavior and occupy relatively large geographic ranges. We can also use this dataset to identify darter species and clades more likely to exhibit phylogenetic patterns consistent with genetic exchange between species. However, the frequency of species involvement in hybrid crosses reported in our dataset is not a direct predictor of those species involved with introgression resulting from hybridization, because evidence of genetic exchange is found in darter species with few or no hybrid records in this dataset.

Variable degrees of hybrid fitness and ability to backcross to parental species can lead to a situation where a pair of frequently hybridizing species may be less likely to exchange material than an infrequently hybridizing pair. Consider the hybrid crosses *P. caprodes* X *P. maculata* (N=28 records) and *P. sciera* X *P. sipsi* (N=1 record). Several molecular studies of *Percina* and Percidae (Near 2002, 2008; Near and Benard 2004; Sloss et al. 2004; Song et al. 1998; Williams et al. 2007) have found no evidence for introgression between *P. caprodes* and *P. maculata*. A molecular phylogenetic analysis of the Bridled Darter clade (*P. sipsi*, *P. smithvanizi* and *P. kusha*) using mtDNA gene sequences indicated that all sampled *P. sispsi* specimens were fixed with introgressed *P. sciera* mitochondrial haplotypes (Williams et al. 2007). This contrast illustrates that the frequency of hybridization between any two species is not predictive of the probability of introgression.

Most of the specimens included here were collected during normal ichthyological sampling such as distribution studies (Keck and Etnier 2005), determining the conservation status of certain species (Lang et al. 2005), or for collection of material for taxonomic and biogeographic research (Page and Near 2007). Therefore, the frequencies of hybrid crosses should not be overly biased unless there is a heavy bias in sampling towards species involved with hybrid crosses or habitats conducive to hybridization. A large temporal bias exists, because these samples represent a minuscule portion of the evolutionary history of these organisms. During the evolutionary past environmental disturbance and range expansions or contractions would have profound effects on the frequency of hybridization. We have a snapshot of current hybridization and our dataset underestimates the full extent of hybridization in the evolutionary history of darters, because it does not include hybrid crosses that were present in the past. This temporal bias in sampling results in instances of introgression, but no contemporary hybridization observed between the parental species. For example, a recent molecular phylogenetic analysis of the *E. blennioides* clade found extensive evidence that *E. blennius* is completely fixed for a set of mitochondrial haplotypes that originated from *E. blennioides* through introgression (Piller et al. 2008); however, there are no known hybrid specimens involving these two species, and the "native" *E. blennius* mtDNA genome is apparently extinct.

Despite the limits of this dataset there are instances of introgression that correlate with the results of this study. The frequent hybridization of *E. caeruleum* and *E. spectabile* clade species has led to the introgression and complete replacement of mitochondrial genomes originating from *E. caeruleum* into the *E. spectabile* clade species *E. uniporum* (Lang and Mayden 2007; Ray et al. 2008). Evidence of introgression among *Nothonotus* species is limited to a hybrid zone with limited directional

introgression between *N. camurus* and *N. chlorobranchius* identified in an analysis of allozymes (Eisenhour 1995). However, three phylogenetic investigations of *Nothonotus* using mitochondrial and nuclear DNA sequence data, allozymes, and external morphological characters (Keck and Near 2008; Near and Keck 2005; Wood 1996) did not find evidence of introgression between *N. camurus* and *N. chlorobranchius* or in the most frequently involved member of the clade, *N. rufilineatus*. These contrary results may be due to inadequate taxon sampling. Eisenhour's (1995) study was directed at resolving relationships between *N. camurus* and *N. chlorobranchius* and included many more individuals of these species than any of the other *Nothonotus* phylogenetic investigations.

CONCLUSIONS

The frequency of hybrid occurrence and the number of unique crosses in darters are much higher than previously reported, but observed patterns fit previously reported patterns of negative associations with increasing phylogenetic divergence and complexity of breeding behavior. Our analysis of darter hybrid specimens identifies species with large geographic ranges, relatively high abundances, and egg burying reproductive behavior as being involved with the greatest number of hybrid crosses. As shown by the comparisons of this dataset to molecular phylogenetic studies, the predictive ability of frequency data of natural darter hybrid crosses has limitations due to temporal bias in sampling and the unknown likelihood of genetic exchange between species. Varied outcomes of hybridization that include frequent occurrence of hybrids, but no observed introgression, to no observed hybrids and evidence of introgression illustrate a wide variance of RIB efficacy and that hybridization is an evolutionarily persistent process in darters. These attributes highlight darters as a candidate system to study the effect of hybridization on patterns of diversification in animal clades.

CHAPTER 3

Assessing phylogenetic resolution among mitochondrial, nuclear, and morphological datasets in *Nothonotus* darters (Teleostei: Percidae)[1]

ABSTRACT

External morphological characters are the basis of our understanding of diversity and species relationships in many darter clades. The past decade has seen the publication of many studies utilizing mtDNA sequence data to investigate darter phylogenetics, but only recently have nuclear genes been used to investigate darter relationships. Despite a long tradition of use in darter systematics few studies have examined the phylogenetic utility of external morphological characters in darter clades. We present DNA sequence data from the mitochondrial cytochrome *b* (cyt*b*) gene, the nuclear encoded S7 intron 1, and discretely coded external morphological characters for all 20 species in the darter clade *Nothonotus*. Bayesian phylogenetic analyses result in phylogenies that are in broad agreement with previous studies. The cyt*b* gene tree is well resolved, while the nuclear S7 gene tree lacks phylogenetic resolution, node support, and is characterized by a lack of reciprocal monophyly for many of the *Nothonotus* species. The phylogenies resulting from analysis of the morphological dataset lack resolution, but nodes present are found in the cyt*b* and S7 gene trees. The highest resolution and node support is found in the Bayesian combined data phylogeny. Based on our results we propose continued exploration of the phylogenetic utility of external morphological characters in other darter clades. Given the extensive lack of reciprocal monophyly of species observed in the S7 gene tree we predict that nuclear gene sequences may have limited utility in intraspecific phylogeographic studies of *Nothonotus* darters.

INTRODUCTION

Molecular systematic studies have provided species-level phylogenetic hypotheses for several darter clades (Near 2002; Near et al. 2000; Porter et al. 2002; Porterfield et al. 1999; Turner 1997). These molecular phylogenies have provided a basis for comparative studies investigating the role of body size in the genetic structuring of darter populations (Turner and Trexler 1998), the evolution of water column habitat utilization and dichromatism (Near 2002), and the evolution of reproductive isolation (Mendelson 2003b). Substantial differences in species-level molecular phylogenies and traditional morphology-based relationships have been discovered in several darter clades (Near 2002; Near et al. 2000; Porter et al. 2002). External morphology and patterns of male nuptial coloration were the basis for hypotheses of species-level relationships previous to these molecular studies, and morphological characters were often not presented as discretely coded character states and hypothesized relationships were not

[1] A slightly modified version of this chapter has been published as: Keck, B.P. and T.J. Near. 2008. Assessing phylogenetic resolution among mitochondrial, nuclear, and morphological datasets in *Nothonotus* darters (Teleostei: Percidae). *Molecular Phylogenetics and Evolution* 46:708-720. My contributions to this manuscript include: 1) expanding ideas, 2) most of the data collection and analyses, 3) much of the writing.

typically presented as trees estimated using phylogenetic methods (Page 1974b; Williams 1975; Zorach 1972; Zorach and Raney 1967). Determination of the agreement among morphological and molecular phylogenetic analyses for most darter clades is constrained by the lack of datasets containing discretely coded morphological character states.

Nothonotus is a clade of 20 described darter species (Perciformes: Percidae) with a most recent common ancestor (MRCA) that has been dated close to 24 million years ago using time-calibrated mtDNA gene trees (Near and Keck 2005). Unlike most other darter clades the diversity of external morphological and male nuptial coloration characters among *Nothonotus* species has been coded as discrete character states and used in maximum parsimony analyses (Etnier and Williams 1989; Wood 1996). The availability of molecular and morphological phylogenies that include specimens of all recognized *Nothonotus* species provides an opportunity to investigate the agreement of phylogenetic hypotheses inferred from morphological characters traditionally used in darter systematics with those resulting from analysis of aligned DNA sequence data.

Figure 3.1 shows the previous phylogenetic hypotheses of *Nothonotus* species based on external morphology and male nuptial color patterns (Etnier and Williams 1989), a combination of allozyme, external morphological, and behavioral characters (Wood 1996), and mitochondrial cytochrome *b* gene sequences (Near and Keck 2005). All of these phylogenetic hypotheses include a resolved clade of egg-guarding species referred to as the *N. maculatus* species clade and includes *N. aquali*, *N. maculatus*, *N. microlepidus*, *N. sanguifluus*, *N. vulneratus*, and *N. wapiti* (Etnier and Williams 1989; Page 1985; Zorach and Raney 1967). In addition, these phylogenetic hypotheses depict the Ozark endemic *N. juliae* as the sister species to all other *Nothonotus* species and phylogenies based on allozyme and mtDNA sequence data result in a sister relationship between *N. acuticeps* and the Mobile Basin endemic *N. jordani* species clade (*N. chuckwachatte, N. douglasi, N. etowahae, and N. jordani*; Fig. 3.1). Despite appreciable

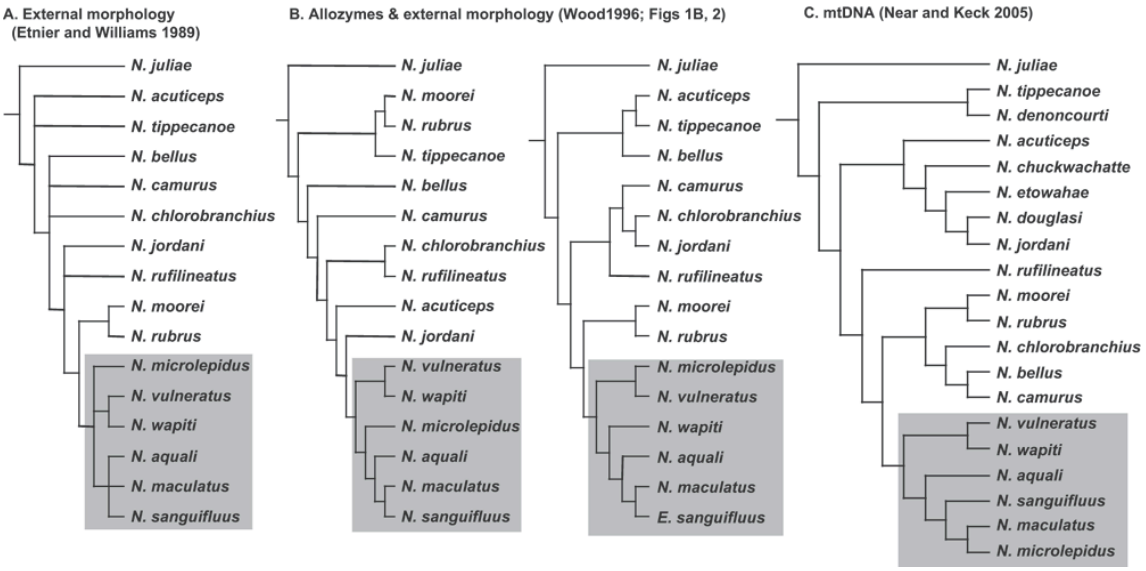

Fig. 3.1. Previous phylogenetic hypothesis for *Nothonotus*.

agreement among these phylogenetic hypotheses some areas of disagreement and regions of poor phylogenetic resolution remain. All previous hypotheses of *Nothonotus* phylogeny are based on phylogenetic datasets that are less than ideal. For instance, the most recent phylogenetic analyses have been based entirely on mtDNA gene sequences and carry all of the caveats of a single gene phylogeny, in particular the biological reality for potential conflict between the true species phylogeny and a single gene tree (Hudson 1992; W.P. Maddison 1997).

The classification of *Nothonotus* has recently been questioned as a result of phylogenetic studies of darters using mtDNA gene sequences (Near and Keck 2005). From the mid-1950's to the present day all darter species are classified into four genera, *Ammocrypta*, *Crystallaria*, *Etheostoma*, and *Percina* (Bailey and Etnier 1988; Bailey and Gosline 1955; Bailey et al. 1954; Boschung and Mayden 2004; Jenkins and Burkhead 1994; Kuehne and Barbour 1983; Page 1981, 1983; Simons 1991). In a discussion of the validity of *Ammocrypta* (including *Crystallaria*), *Etheostoma*, and *Percina* Bailey et al. (1954) provide clear morphological synapomorphies for *Ammocrypta* and *Percina*; however, no apomorphies for *Etheostoma* are identified and the group is defined as containing any darter species that is not *Ammocrypta*, *Crystallaria*, or *Percina* (Bailey et al. 1954; Page 1981). *Nothonotus* species have historically fallen into this rubric and since Bailey et al. (1954) *Nothonotus* has been classified as a subgenus of *Etheostoma* (Bailey and Etnier 1988; Boschung and Mayden 2004; Etnier and Starnes 1993; Kuehne and Barbour 1983; Page 1981). Phylogenetic studies using mtDNA gene sequences have consistently resulted in a non-monophyletic *Etheostoma*, because species of *Nothonotus* and *Allohistium* are more closely related to other darter clades (Sloss et al. 2004; Song et al. 1998). Based on these results, Near and Keck (2005) recommended the treatment of *Nothonotus* as a distinct clade and not a subgenus of *Etheostoma*. Lang and Mayden (2007) obtain a similar pattern of *Etheostoma* non-monophyly from a phylogeny inferred from mtDNA gene sequences, but their phylogenetic analysis of the nuclear encoded S7 intron 1 resulted in a monophyletic *Etheostoma* with weak node support. Lang and Mayden (2007) suggest this latter result should motivate the recognition of *Nothonotus* as a sub-clade of *Etheostoma*. However, our conclusions are based on the premise that the evolutionary history of a clade is described by any number of datasets and each can offer strengths and weaknesses for elucidating the phylogenetic relationships of the lineage. Given the distinct evolutionary history of *Nothonotus*, with strongly supported mtDNA inferred relationships closer to other darter lineages and weakly supported nuclear DNA inferred relationships with *Etheostoma,* and the distinct lack of morphological apomorphies for an inclusive *Etheostoma*, we suggest that *Nothonotus* be recognized as a distinct clade and not a subgenus of *Etheostoma*.

To investigate the agreement and degree of resolution between phylogenies inferred from different types of data and to determine the uniqueness of phylogenies inferred from combined data analyses, we collected mitochondrial and nuclear gene sequences from specimens of all *Nothonotus* species and utilized coded morphological character states used in previous phylogenetic studies. Combining morphological data sets with molecular data sets in maximum likelihood based analyses has become an option with the description of the Mk model by Lewis (2001). The results obtained using the Mk model in maximum likelihood phylogenetic analyses are typically very similar to

those obtained using parsimony optimality criteria (Lewis 2001; Nylander et al. 2004). A common assumption is that the nucleotide data will overwhelm any signal from morphological data in combined data phylogenetic analyses, but the combined analysis of morphological and molecular data generally results in phylogenies with higher resolution and support (Engstrom et al. 2004; Nylander et al. 2004; Wahlberg et al. 2005). Our analyses aim to provide insight as to the phylogenetic utility of external morphological and pigmentation characters traditionally used to investigate species level relationships in darter clades. Additionally, we discuss the prospect of using nuclear gene DNA sequence data to resolve phylogenetic relationships among closely related darter species and investigate phylogeographic patterns within species.

METHODS

All specimens were captured using seine nets or a backpack electrofishing unit (Table B.1). Tissue biopsies were preserved in 100% ethanol and stored at 4° C. Voucher specimens were fixed in 10% formalin for at least seven days, washed in tap water for two days, and transferred to 70% ethanol for long-term preservation. All specimens were deposited into either the University of Tennessee Research Collection of Fishes (UT) or the Yale Peabody Museum of Natural History fish collection (YPM). Darters are classified in Percidae and we sampled two non-darter percids and nine other darter species from *Ammocrypta*, *Crystallaria*, *Etheostoma*, and *Percina* as outgroup species (Table B.1).

Nucleic acids were extracted from tissue biopsies using the Qiagen DNAeasy DNA kits following the manufacturer's protocol. The mtDNA encoded cytochrome *b* (cyt*b*) gene and the first intron of the nuclear encoded S7 ribosomal protein were amplified using PCR with primers and conditions outlined in Near et al. (2000) and Chow and Hazama (1998). Amplification products resulting from successful PCR were prepared for sequencing using Qiagen Qiaquick kits or by digesting with 1.0 unit of Exonuclease I and shrimp alkaline phosphatase and incubated for 15 minutes at 37 ºC and 20 minutes at 80 ºC. Treated PCR products were used as template for DNA sequencing that was performed by the DNA Sequencing Facility on Science Hill at Yale University or WM Keck Foundation Biotechnology Resource Laboratory at Yale University. Contiguous sequences were assembled from individual sequencing reactions using the computer program Sequencher version 4.5 (Gene Codes, Ann Arbor, MI, USA). Alignments of mtDNA cyt*b* gene were performed by eye and the S7 intron was aligned using the computer program MUSCLE (Edgar 2004). Four data partitions were identified: three codon positions in the protein coding cyt*b* gene, and a single partition for the S7 intron. The optimal maximum likelihood model for each partition was determined with AIC as executed in the computer program Modeltest 3.0 (Posada and Crandall 1998).

Discretely coded character states for external morphology and male nuptial coloration published in Etnier and Williams (1989) and Wood (Wood 1996)were entered into MacClade 4.0 (D.R. Maddison and Maddison 2000). Character state coding for outgroup species followed those presented in Etnier and Williams (1989), examination of photographs of specimens from our field work, and examination of preserved museum specimens (Table B.2).

Phylogenetic hypotheses were generated with partitioned mixed-model Bayesian analyses (Ronquist and Huelsenbeck 2003) with posterior probabilities estimated using metropolis-coupled Markov chain Monte Carlo (Huelsenbeck et al. 2001; Larget and Simon 1999). We performed separate analyses for the cyt*b*, S7 intron, and morphological datasets. The optimal maximum likelihood models identified for each of data partitions in the molecular data were used in the computer program MrBayes 3.1 (Ronquist and Huelsenbeck 2003). Morphological characters were treated as "standard" in MrBayes and used the Mk model (Lewis 2001). This model allows for there to be *k* number of discrete character states and the rate of character change to be equal or to vary. We utilized a gamma distribution of among character rate variation that allowed a different rate of change for the individual morphological characters (Muller and Reisz 2006; Nylander et al. 2004). Four sets of Bayesian analyses were run, one for each molecular dataset and morphology, and an analysis where all three datasets were combined. Only a single operational taxonomic unit (OTU) was scored for each species in the phylogenetic analyses using the morphological data; however, multiple OTUs were scored for each species (except *N. maculatus*) in the combined data analyses. In each analysis MrBayes 3.1 was run for 6.0×10^6 generations to ensure convergence of the MC3 algorithm and the number of generations discarded as the burn-in was determined by plotting the maximum likelihood score versus the number of generations. The posterior probabilities of nodes in the phylogeny were calculated from the post burn-in trees. All resulting phylogenetic hypothesis were rooted with 11 outgroup species (Table B.1).

In addition to the Bayesian analysis we used maximum parsimony to analyze the morphological data. The most parsimonious trees were found using PAUP* 4.0 (Swofford 2003) with heuristic tree searches and 100 addition sequence replicates. All characters were treated as unordered and node support was assessed with a bootstrap analysis using 2,000 pseudoreplicates.

RESULTS

Taxon sampling for this phylogenetic analysis included 90 individuals sampled from all 20 *Nothonotus* species. The only species sampled with a single specimen was *N. maculatus* (Table B.1). The aligned cyt*b* sequences consisted of 1,140 base pairs (b.p.) and did not include any insertions or deletions. The size of the nuclear S7 ribosomal protein intron 1 ranged from 516 to 530 b.p. among *Nothonotus* species and the length of the S7 ribosomal protein intron 1 alignment was 549 b.p. including gaps. All new sequences were submitted to Genbank: accession numbers EU094658–EU094820.

Intraspecific uncorrected pairwise genetic distances were less than 1.0% for most species, but five species had a maximum genetic distance higher than 1.0% for the cyt*b* gene and seven species had maximum intraspecific genetic divergences exceeding 1.0% for S7 (Table B.3). Maximum intraspecific genetic distances at the S7 locus were much higher than the maximum genetic distances observed for mitochondrial cyt*b* in *Nothonotus aquali*, *N. douglasi*, and *N. microlepidus* (Table B.3).

Bayesian analysis of the cyt*b* alignment resulted in a set of posterior trees that were fairly well resolved and similar to previous analyses of *Nothonotus* phylogeny (Fig. 3.2). The node representing the MRCA of *Nothonotus* and most internal nodes in the cyt*b* phylogeny were supported with significant Bayesian posterior probabilities. A notable

exception was the MRCA of the Mobile Basin endemic *N. jordani* clade (*N. jordani*, *N. etowahae*, *N. douglasi*, and *N. chuckwachatte*) that was supported with a non-significant Bayesian posterior probability, but the MRCA node of *N. acuticeps* and the *N. jordani* species group was supported with a significant posterior probability. Five of the 19 *Nothonotus* species sampled with multiple specimens were not reciprocally monophyletic in the cyt*b* Bayesian phylogeny. In two cases haplotypes from sister species pairs (*N. bellus*-*N. camurus* and *N. sanguifluus*-*N. microlepidus*) did not sort to exclusive lineages (Fig. 3.2). The three specimens sampled from *N. wapiti* were reciprocally monophyletic and nested in a paraphyletic group of *N. vulneratus* haplotypes.

The Bayesian phylogeny inferred from the nuclear encoded S7 ribosomal protein intron 1 was much less resolved than the cyt*b* phylogeny with only three internal nodes supported with significant posterior probabilities (Fig. 3.3). The alleles sampled from individual *Nothonotus* species were reciprocally monophyletic in only five of the 19 species sampled with two or more individuals. This was not limited to paraphyly between sister species as seen in the cyt*b* phylogeny, but alleles sampled from *N. douglasi*, *N. aquali*, *N. rufilineatus*, and *N. camurus* were widely distributed throughout the S7 Bayesian phylogeny (Fig. 3.3).

Phylogenetic analysis of the 12 discretely coded morphological characters resulted in very little resolution among *Nothonotus* species (Fig. 3.4). Despite the lack of resolution, both analyses resulted in phylogenies that placed *N. juliae* as the sister species to all other *Nothonotus*, a relationship consistent with the phylogenies inferred from cyt*b* and S7 (Figs. 3.2 and 3.3). Maximum parsimony analysis found 611 most parsimonious trees with 24 inferred changes. The strict consensus of these trees is presented in Figure 3.4. The only node supported with a bootstrap score ≥ 75 in the maximum parsimony tree was the node depicting *N. tippecanoe* and *N. denoncourti* as sister species (Fig. 3.4). No nodes were supported with significant Bayesian posterior probability scores.

The Bayesian analyses of the combined molecular and morphological data resulted in a phylogeny similar to the cyt*b* Bayesian phylogeny with regard to specific relationships and degree of resolution (Fig. 3.5). Similar to the cyt*b* phylogeny the combined data phylogenetic analyses resulted in N. *juliae* as the sister species to all other *Nothonotus* species, *N. tippecanoe* and *N. denoncourti* as sister species, and monophyly of the six species in the *N. maculatus* clade that exhibit male parental care (Fig. 3.5). The monophyly of the Mobile Basin endemic *N. jordani* species clade was not supported in greater than 50% of the post burn in Bayesian phylogenies; however, the monophyly of clade containing the species in the *N. jordani* clade and the upper Tennessee River endemic *N. acuticeps* was supported with a significant posterior probability. The MRCA of the clade containing *N. camurus*, *N. bellus*, and *N. chlorobranchius*, and the MRCA of *N. wapiti* and *N. vulneratus* (exclusive of *N. vulneratus* E) were supported with significant posterior probabilities, but these nodes were not supported in the cyt*b* phylogeny (Figs. 3.2 and 3.5). In the combined data phylogeny three of the 19 *Nothonotus* species with more than one specimen sampled were not reciprocally monophyletic. Both *N. camurus* and *N. bellus* were reciprocally monophyletic in the

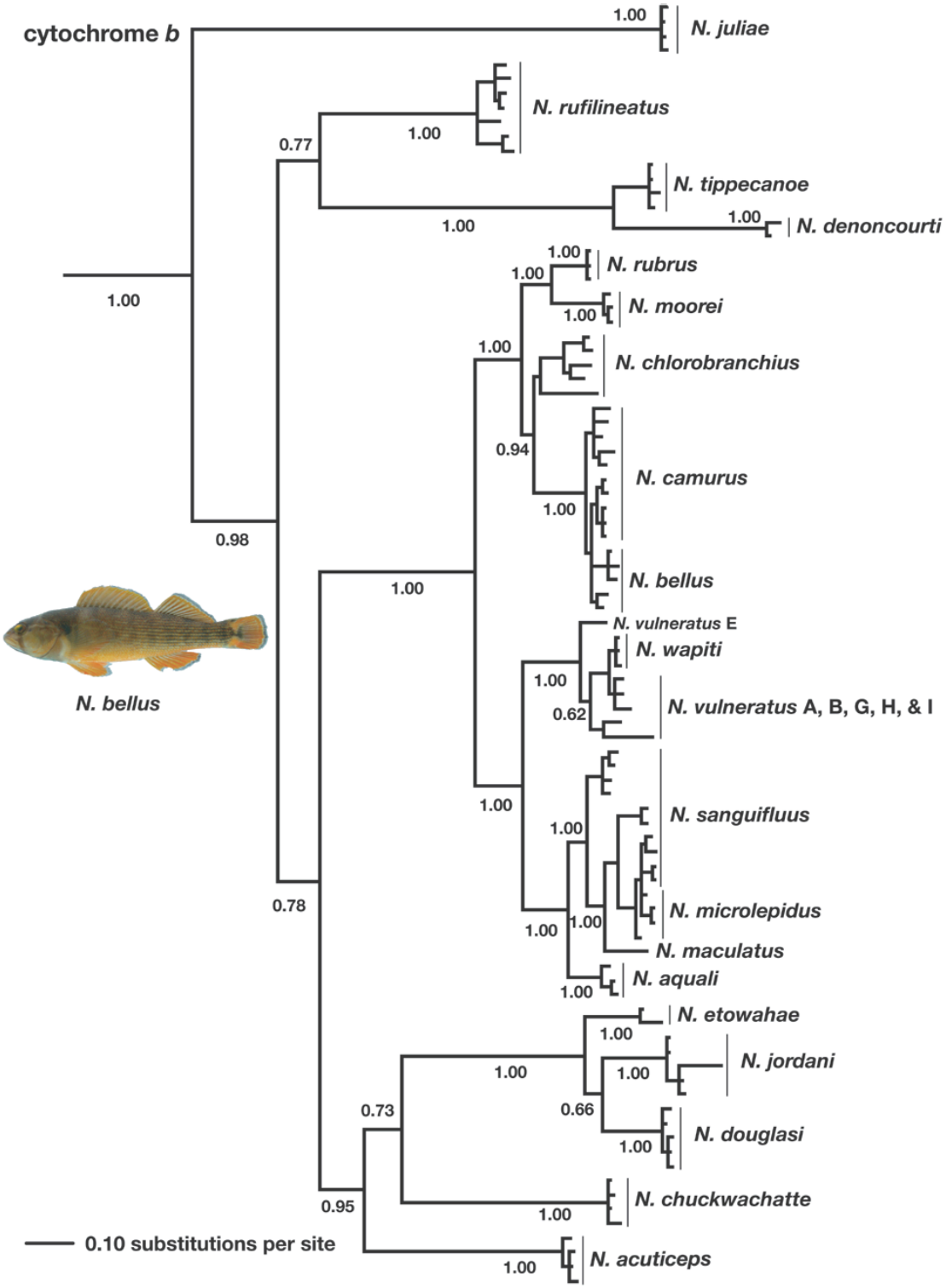

Fig. 3.2. Phylogeny of *Nothonotus* resulting from Bayesian analysis of mitochondrial cytochrome *b* gene sequences. Numbers at nodes represent Bayesian posterior probabilities.

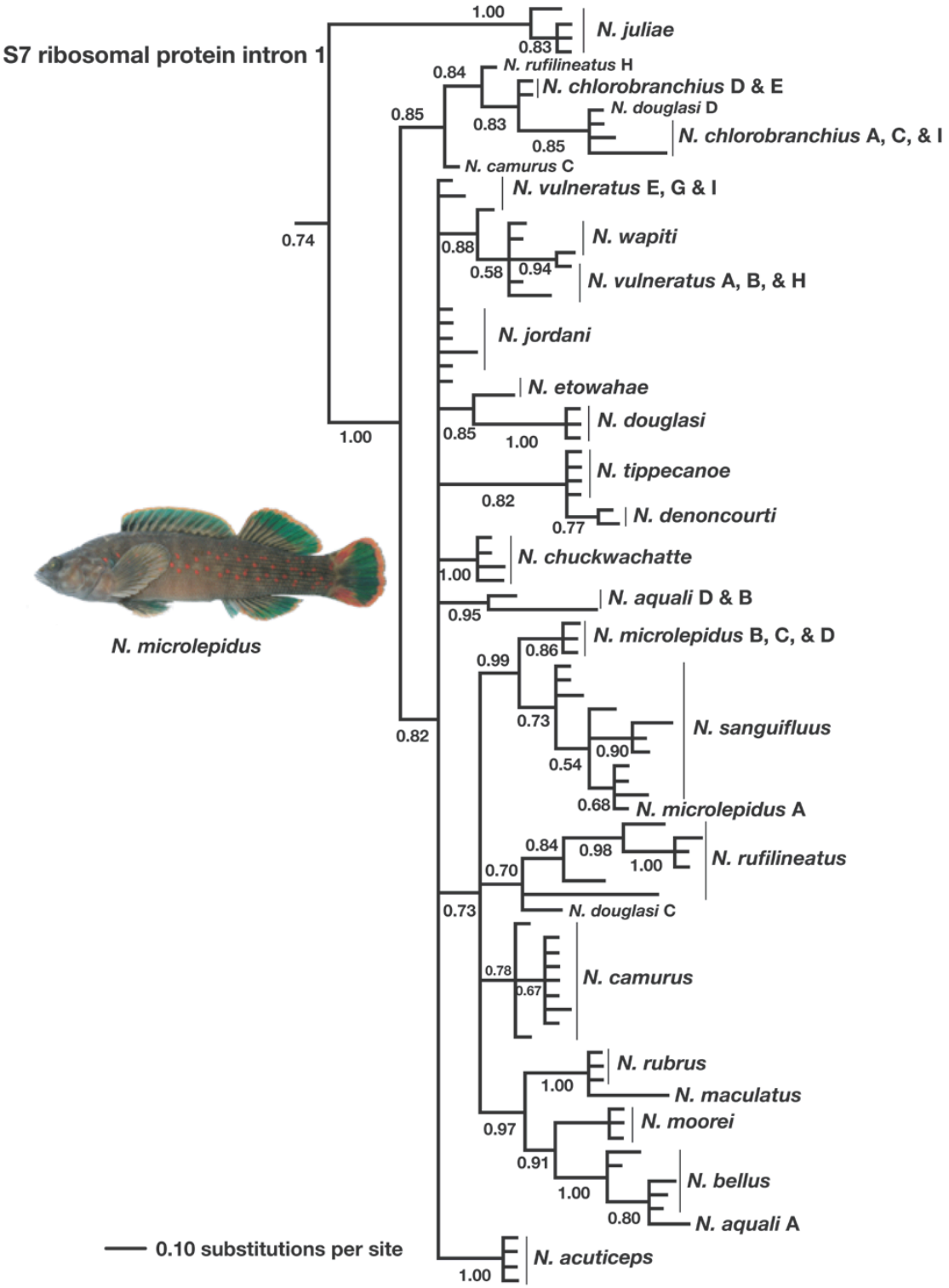

Fig. 3.3. Phylogeny of *Nothonotus* resulting from Bayesian analysis of nuclear encoded S7 ribosomal protein intron 1 gene sequences. Numbers at nodes represent Bayesian posterior probabilities.

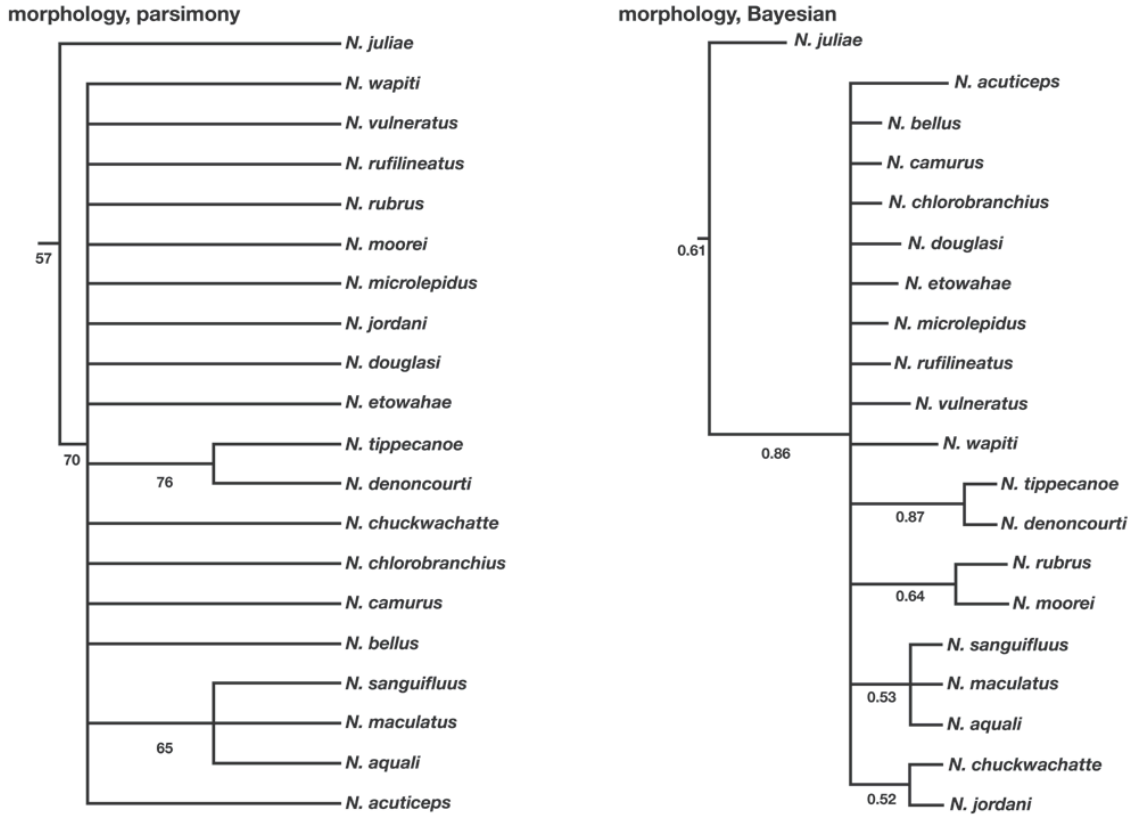

Fig. 3.4. Phylogenies for *Nothonotus* species resulting from maximum parsimony and Bayesian analyses of external morphological characters. Numbers at nodes in the parsimony phylogeny represent percent recovery in bootstrap analysis, and those in the Bayesian phylogeny represent posterior probabilities.

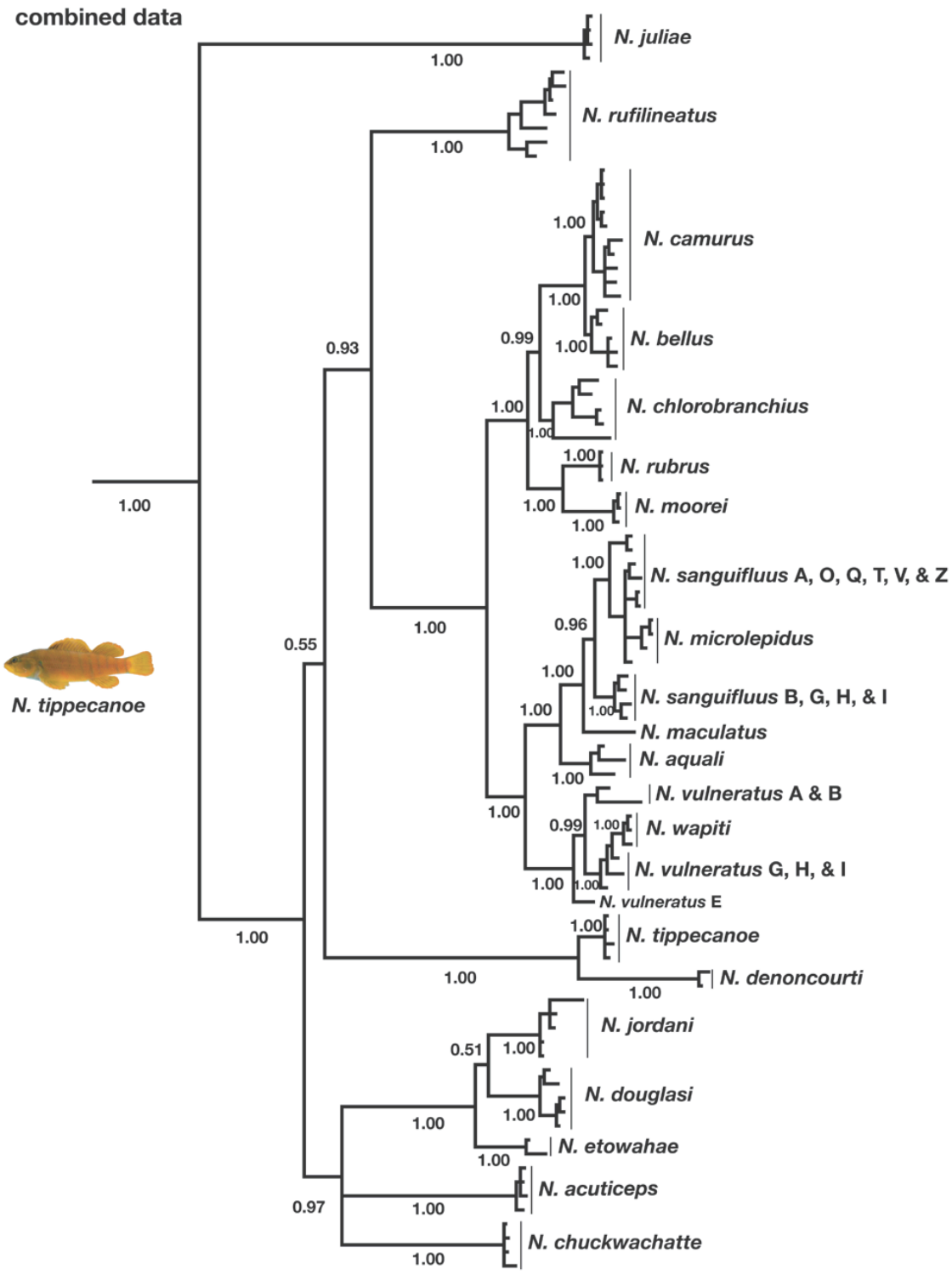

Fig. 3.5. Phylogeny of *Nothonotus* resulting from Bayesian analysis of combined mitochondrial cytochrome *b*, nuclear encoded S7 ribosomal protein intron 1 gene sequences, and discretely coded external morphological characters. Numbers at nodes represent Bayesian posterior probabilities.

combined data phylogenetic analysis; however, haplotypes sampled from these two species were not reciprocally monophyletic in the cyt*b* phylogeny.

DISCUSSION

The phylogenetic analyses for *Nothonotus* presented in this study used one of the most comprehensive datasets ever compiled to investigate relationships among species in a darter clade. Despite the large amount of data compiled for this investigation the results of our phylogenetic analyses are strikingly similar to previous efforts using external morphology, behavioral characters, and allozyme variation shown in Figure 3.1 (Etnier and Williams 1989; Wood 1996). In particular, all phylogenetic analyses of *Nothonotus* have resulted in *N. juliae* as the sister species of all other *Nothonotus* species, a sister species relationship between *N. moorei* and *N. rubrus*, and a clade containing the species that exhibit parental care through male egg guarding. The exceptions are the phylogenies resulting from analysis of the S7 intron and external morphology datasets that lacked resolution, including the monophyly of species that exhibit male parental care (Figs. 3.3 and 3.4).

Does the substantially different phylogenetic resolution obtained with the mitochondrial cyt*b* gene sequences and either the nuclear gene sequences or external morphology provide any direction for studying species-level relationships in other darter clades? Few published studies have examined the phylogenetic utility of external morphological characters in resolving relationships among darter species. Braasch and Mayden (1985) used 46 discrete external morphological characters to examine relationships among species in the darter clade *Catonotus*. The phylogenies were completely resolved and subsequent phylogenetic analyses of *Catonotus* using cyt*b* DNA sequences found broad agreement with Braasch and Mayden's (1985) morphological phylogeny (Porterfield et al. 1999). Page (1974b; 1981) combined discrete and continuous characters from larger numbers of darter species for phenetic analyses to determine if predefined genera and subgenera clustered in the resulting phenograms. The results of these analyses were inconclusive, as genera and subgenera failed to cluster. However, it was not clear if such relationships resulted from differences at discrete or continuous characters. Unfortunately, the most extensive analysis of darter phylogeny using discrete morphological characters is a Ph.D. dissertation that has been awaiting publication for over a decade (K.A. Shaw 1996). In this study, Shaw (1996) presents discrete data from most species of *Etheostoma* (including *Nothonotus*) for 141 characters that include external morphology, male nuptial coloration, osteology, and behavior. The phylogenies are well resolved and in many cases unique; however, the unpublished status of this work constrains the scientific community's access to the data and phylogenetic hypotheses.

It is not surprising that the *Nothonotus* phylogenies inferred from the external morphological data were unresolved, especially considering there were only 12 characters available for analysis (Table B.2, Fig. 3.4). However, it is important to note that despite the lack of global resolution the nodes present in the morphological trees were congruent with the molecular phylogenies, particularly the cyt*b* analysis (Figs. 3.2 and 3.3). For example, *N. juliae* as the sister species to all other *Nothonotus* species, *N. tippecanoe* and *N. denoncourti* as sister species, and *N. rubrus* and *N. moorei* as sister

species are relationships found in the morphological and cyt*b* phylogenies (Figs. 3.2 and 3.4), as well as several other previously published studies of *Nothonotus* taxonomy and phylogeny (Etnier and Williams 1989; Kinziger et al. 2001; Raney and Suttkus 1966; Stauffer and Van Snik 1997; Wood 1996). Despite the lack of phylogenetic resolution the morphological characters do provide evidence for at least three nodes in the *Nothonotus* phylogeny that is independent of the cyt*b* and S7 gene trees.

When compared to the nuclear gene S7 phylogeny (Fig. 3.3) the higher phylogenetic resolution and greater frequency of reciprocally monophyletic species in the cyt*b* phylogeny is expected, because ancestral genetic polymorphisms will sort faster for mitochondrial haplotypes than nuclear gene alleles (Hudson and Coyne 2002). This is due to the differences in the effective population sizes across genomes, where the mitochondrial genome has an effective population size that is one quarter that of a given set of linked nuclear autosomal loci (Avise et al. 1988; Birky et al. 1983; Moore 1995). Despite the expected difference in resolution between mitochondrial and nuclear gene trees, mitochondrial gene trees can be incongruent with the species tree due introgression facilitated by interspecific hybridization (Avise 1994; W.P. Maddison 1997) and comparing mitochondrial and nuclear gene trees provides a way to identify instances of introgression (Bachtrog et al. 2006; K.L. Shaw 2002). Hybridization and introgression between *N. chlorobranchius* and *N. camurus* has been documented using allozyme data in an area of secondary contact between these two species in the Nolichucky River in Tennessee (Eisenhour 1995); however, we did not detect any patterns consistent with introgression in our limited sampling of these two species.

We propose that the lack of reciprocal monophyly observed in both the nuclear S7 and mitochondrial cyt*b* phylogenies is due to internodes in the *Nothonotus* phylogeny that are short when scaled by the ancestral effective population sizes along specific branches. This lack of sorting is extensive in the S7 phylogeny (Fig. 3.3), but limited to sister species contrasts in the cyt*b* tree (Fig. 3.2). A previous study using external fossil calibrations to estimate molecular divergence times with a *Nothonotus* cyt*b* phylogeny resulted in ages that were all less than 1.5 million years for each MRCA node containing species that are not reciprocally monophyletic in the cyt*b* phylogeny (Near and Keck 2005), indicating that short branches in evolutionary time coupled with relatively large effective populations sizes may explain the lack of mtDNA haplotype sorting for these *Nothonotus* species. However, the sister species *N. microlepidus* and *N. sanguifluus*, while non-monophyletic, exhibit similar patterns in both the cyt*b* and S7 phylogenies (Figs. 3.2 and 3.3). The MRCA node for this species pair, and *N. maculatus*, also dates to approximately 1.4 million years suggesting that these species may have distinctly different population sizes or dynamics than the other sister species pairs with MRCA nodes of the same age.

The results from our analyses have provided a valuable phylogenetic hypothesis of *Nothonotus* that includes data from a mitochondrial gene, a nuclear gene, and external morphology (Fig. 3.5). We predict that phylogenies of other darter clades using limited external morphological characters will provide much less resolution than mtDNA sequence data. Despite lower resolution these morphological phylogenies can provide independent verification for key nodes in the phylogeny. Nuclear gene data has only recently been applied to darter phylogenetics (Lang and Mayden 2007; Morrison et al.

2006; Page et al. 2003), but our study is the first to find extensive lack of reciprocal monophyly among species (Fig. 3.3). The utilization of DNA sequences from multiple nuclear genes is clearly becoming the state of the art in molecular systematics and we envision that such data will drive future investigations of darter phylogenetics. Despite the lack of resolution in the nuclear S7 gene tree, combination with the cyt*b* and morphological datasets resulted in a tree that was more resolved with slightly better node support than the cyt*b* phylogeny. This indicates that in addition to identifying instances when the mitochondrial gene tree can be misleading the addition of the nuclear gene dataset to mtDNA datasets can improve the resolution of species level relationships. On the other hand, our study indicates that the prospect of resolving intraspecific relationships or phylogeographic patterns among most *Nothonotus* species using nuclear gene DNA sequences is not very promising. This conclusion is supported by the finding that a very long divergence time between sister species is needed to observe reciprocal monophyly for large numbers of autosomal loci (Hudson and Coyne 2002). In cases such as *Nothonotus* where introgression does not appear to obscure the mitochondrial gene tree, perhaps the best prospect for examining patterns of intraspecific phylogeography will come from mitochondrial gene data.

CHAPTER 4

Geographic and temporal aspects of mitochondrial replacement in *Nothonotus* darters (Teleostei: Percidae: Etheostomatinae)[1]

ABSTRACT

A rapidly growing number of molecular phylogenetic studies are identifying mitochondrial replacement through hybridization in animal clades, but there has been little insight on the geographic and temporal patterns of mitochondrial introgression. In this study we present a phylogenetic analysis of mitochondrial and nuclear DNA sequences collected from *Nothonotus* darter species and reveal extensive mitochondrial DNA replacement in *N. rufilineatus*, a species endemic to the Tennessee and Cumberland River drainages that drain the biodiverse Eastern Highlands of North America. Using phylogenetic tree topologies, allele networks, AMOVA, and distributions of minimum genetic distances we reveal that the mitochondrial genome of *N. rufilineatus* is replaced by that of other *Nothonotus* species that are sympatric in different river drainages. In the Cumberland River drainage populations of *N. rufilineatus* harbor *N. camurus* mitochondrial genomes and the upper Tennessee River populations of *N. rufilineatus* contain *N. chlorobranchius* mitochondrial genomes. Our analyses indicated that *N. rufilineatus* is acting as a 'conduit species', facilitating the introgression of *N. chlorobranchius* mitochondrial genomes into *N. camurus* in upper Tennessee River populations. In *Nothonotus* there is asymmetric mitochondrial replacement, the ranges of the mitochondrial donor species are usually larger than those of the recipient species, and when multiple species are involved in mitochondrial replacement, donor species and recipient species are in exclusive clades. We identify several mechanisms potentially responsible for the observed patterns and suggest some experimental tests to assess their relative contributions.

INTRODUCTION

Introgression occurs in lineages across the Tree of Life and has the potential to alter the evolutionary trajectories of clades (Arnold 2006; P. R. Grant et al. 2004; Seehausen 2004). Identification of the geographic and temporal aspects of introgression provides a basis to explore the mechanisms that facilitate gene flow between species. It is also important to identify instances of introgression for comparative studies, because introgressed genes may disrupt inferences of phylogeny using molecular genetic characters (Fig. 4.1)(Avise 2004; Chan and Levin 2005; Funk and Omland 2003; P. R. Grant et al. 2005; W.P. Maddison 1997; Moore 1995; K.L. Shaw 2002; Sota and Vogler 2001). Studies of natural introgression often focus on describing exchange between a few species (Good et al. 2008; Hofman and Szymura 2007; Linnen and Farrell 2008; Maletzky et al. 2008; Schelly et al. 2006; Vigfusdottir et al. 2008; Yanchukov et al. 2006), describing correlations between habitat,

[1] A modified version of this chapter to be submitted as: Keck, B.P. and T.J. Near. Geographic and temporal aspects of mitochondrial replacement in *Nothonotus* darters (Teleostei: Percidae: Etheostomatinae). *Evolution*. My contributions to this manuscript include: 1) development of ideas, 2) data collection and analyses, 3) most of the writing.

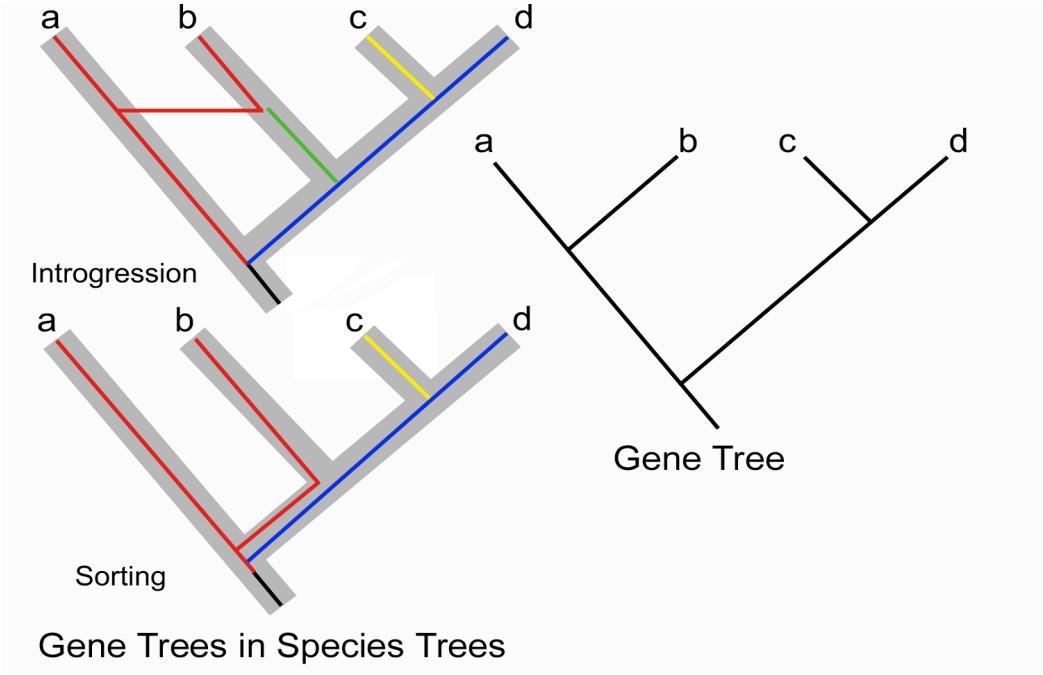

Fig. 4.1. Gene trees in species trees. Hypothetical genealogies (thin branches) in species trees (thick gray branches) on the left showing how introgression (top) and stochastic sorting (bottom) can result in a gene tree (on right) with a different topology than the true species tree.

behavior, or morphology and the extent of introgression (Arnold et al. 2008; Bouck et al. 2007; Fitzpatrick and Shaffer 2007; Ganem et al. 2008; Lavoue et al. 2008; Ruegg 2008), or examining the geographic and temporal aspects of introgression events between closely related species (Bossu and Near accepted; Carson and Dowling 2006; Cronn et al. 2003; McGuire et al. 2007; Ray et al. 2008; K.L. Shaw 2002). There are fewer studies that have focused on introgression within more inclusive clades, and these have focused on the biased introgression of cytoplasmic genomes (Chan and Levin 2005; Scribner et al. 2001). Given the multitude of examples of introgression, studies comparing patterns of introgression from multiple lineages are conspicuously lacking.

Compared to other vertebrates, hybridization and introgression are relatively common among freshwater teleost fishes (Avise 2004; Campton 1987; C.L. Hubbs 1955; Scribner et al. 2001) and instances of hybridization have been identified in several subclades of darters, a clade of approximately 225 species endemic to freshwater habitats in eastern North America (Bossu and Near accepted; Eisenhour 1995; Page et al. 1992; Piller et al. 2008; Ray et al. 2008; Williams et al. 2007). In the course of investigating the phylogenetic relationships of the darter subclade *Nothonotus* (Keck and Near 2008; Near and Keck 2005), we discovered that mitochondrial haplotypes sampled from *N. rufilineatus* clustered in three distinct regions of the *Nothonotus* phylogeny. One of the clusters agreed with previous hypotheses of the position of *N. rufilineatus* within *Nothonotus* (Etnier and Williams 1989; Wood 1996), but the other two clusters indicated extensive replacement of the *N. rufilineatus* mitochondrial genome by the mitochondrial genomes of the sympatric species *N. camurus* and *N. chlorobranchius*.

Nothonotus is a darter subclade of 20 species that are often strikingly sexually dichromatic, occupy the faster flowing areas of small to larger streams and rivers, and species in the clade exhibit either egg burying or egg guarding behaviors (Etnier and Starnes 1993). The six egg guarding species are monophyletic and known as the *N. maculatus* clade (inclusive of *N. aquali*, *N. maculatus*, *N. microlepidus*, *N. sanguifluus*, *N. vulneratus* and *N. wapiti*). The *N. maculatus* clade species are entirely allopatric with other *N. maculatus* clade species, but sympatric with egg burying *Nothonotus* species. The egg burying *Nothonotus* are often sympatric with other egg burying *Nothonotus* species, and the geographic distribution of *N. rufilineatus*, *N. camurus*, and *N. chlorobranchius* overlap in the Cumberland and Tennessee River drainages in the southeastern United States (Fig. 4.2). *Nothonotus rufilineatus* is endemic to, and abundant in, the Cumberland and Tennessee River drainages, *N. camurus* has a sporadic distribution in these rivers, but is also found in more northern Ohio River tributaries, and *N. chlorobranchius* is restricted to tributaries in the upper Tennessee River drainage that flow through the Blue Ridge physiographic region (Fig. 4.3) (Etnier and Starnes 1993; Lee et al. 1980; Page 1983). *Nothonotus rufilineatus* and *N. camurus* are sympatric in the Cumberland River drainage, and all three species, *N. rufilineatus*, *N. camurus*, and *N. chlorobranhius*, are sympatric in the Upper Tennessee River drainage (Fig. 4.3).

Asymmetric mitochondrial introgression with little or no evidence of introgression of nuclear material has been documented in species from disparately related darter subclades (Bossu and Near accepted; Lang and Mayden 2007; Piller et al. 2008; Ray et al. 2008). Biased introgression of mitochondrial genomes is common in other

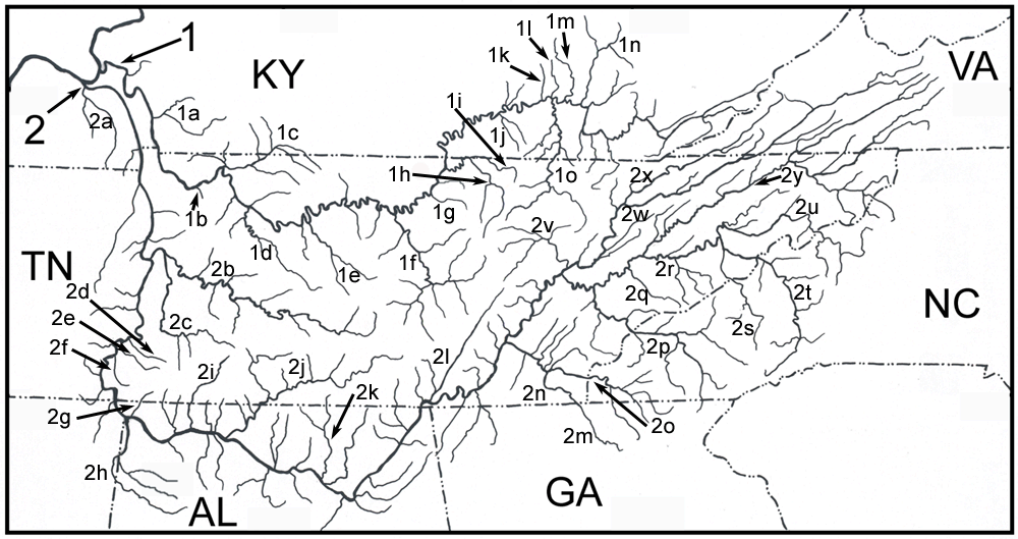

Fig. 4.2. Map of the Cumberland and Tennessee River drainages with tributaries numbered and US States labeled. Key to river numbers: **1**–Cumberland R., **a**–Little R., **b**–Yellow Cr., **c**–Red R., **d**–Harpeth R., **e**–Stones R., **f**–Caney Fork R., **g**–Roaring R., **h**–Obey R., **i**–Wolf R., **j**–Otter Cr., **k**–Fishing Cr., **l**–Pitman Cr., **m**–Buck Cr., **n**–Rockcastle R., **o**–Big South Fork Cumberland R.; **2**–Tennessee R., **a**–Clark's R., **b**–Duck R., **c**–Buffalo R., **d**–Hardin Cr., **e**–Indian Cr., **f**–Horse Cr., **g**–Second Cr., **h**–Cedar Cr., **i**–Shoal Cr., **j**–Elk R., **k**–Flint R., **l**–Sequatchie R., **m**–Taccoa R., **n**–Ocoee R., **o**–Hiwassee R., **p**–Little Tennessee R., **q**–Little R., **r**–Little Pigeon R., **s**–Pigeon R., **t**–French Broad R., **u**–Nolichucky R., **v**–Emory/Obed R., **w**–Clinch R., **x**–Powell R., **y**–Holston R.

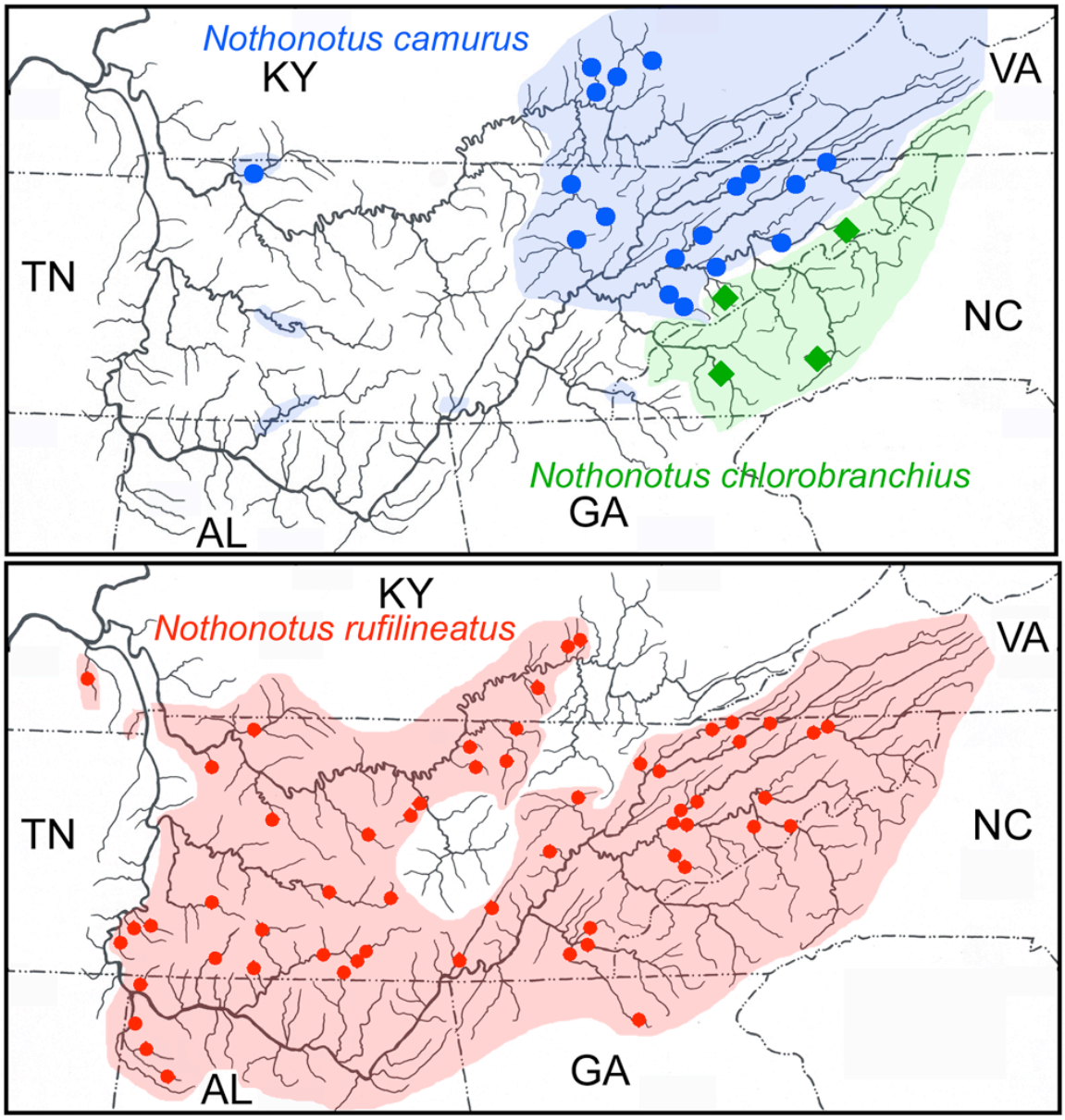

Fig. 4.3. Map of the range and sampling of *Nothonotus camurus*, *N. chlorobranchius*, and *N. rufilineatus* in the Cumberland and Tennessee Rivers. Blue shading indicates the range of *N. camurus* and blue markers indicate sampled localities. *Nothonotus camurus* occurs in much of the Ohio River drainage and several samples were taken from those rivers, but are not shown here. Green shading indicates the range of *N. chlorobranchius* and green markers indicate sampled localities. Red shading indicates the range and red markers indicate sampled localities of *N. rufilineatus*.

teleost fish clades (Scribner et al. 2001), and is a pattern documented in other animal clades (Chan and Levin 2005). Potential mechanisms that result in this observed bias include asymmetric sexual isolation (Coyne et al. 2002; Konishi and Takata 2004; Mendelson 2003a; Wirtz 1999), demographic effects (Takahata and Slatkin 1984), selective sweeps of adaptive mito-types (Doiron et al. 2002), and asymmetric genetic incompatibilities such as Darwin's Corollary to Haldane's Rule (Bolnick et al. 2008; Turelli and Moyle 2007).

Investigating patterns of mitochondrial introgression in darters with respect to geographic distribution of hybridizing species may help to reveal mechanisms that result in asymmetric mitochondrial replacement. For example, comparing the range sizes of darter species that donate mitochondrial genomes with those of the receiving darter species reveals that the donating species' ranges are much larger than those of the receiving species (Bossu and Near accepted; Lang and Mayden 2007; Ray et al. 2008; Williams et al. 2007). This range size discordance was also observed in a study of museum hybrid darter specimens, where the darter species most commonly represented in hybrid specimens also had some of the largest ranges of all darter species (Chapter 2).

The primary objective of this study was to determine the extent of introgression in *Nothonotus* darters, and describe the geographic and temporal aspects of introgression between *Nothonotus* species using phylogenies, haplotype and allele networks, AMOVA, and minimum genetic distances. Specifically, we addressed three questions using a comparative molecular phylogenetic dataset. 1) Is there directional mitochondrial replacement with little to no introgression of nuclear alleles between *Nothonotus* species? 2) Is there disparity between the geographic ranges of the mitochondrial donor and recipient species? 3) When more than two species are involved with mitochondrial replacement, is one species always the donor?

METHODS

Geneologies that are discordant with other genealogies or previous phylogenetic hypotheses may be the result of introgression or stochastic sorting of ancestral polymorphism, and these two processes can result in the same incongruence of gene trees and species trees (Fig. 4.1) (Funk and Omland 2003). Differentiating between these can be difficult unless there is contemporary hybridization between the species in the discordant relationship. Geographic structure to the discordant relationship may also indicate introgression rather than ancestral polymorphism, but disproving one scenario is unlikely. For instance, in Fig. 4.1, if **a** and **b** are sympatric then introgression may be a more likely candidate, but if they are allopatric then stochastic sorting seems more likely.

The ability to infer the geographic origin of introgressed alleles depends on the genetic similarity of the alleles transferred between the hybridizing species. The most confident assessments of introgression are from areas where there is ongoing or recent hybridization that results in the presence of identical, or very similar alleles in both species (Good et al. 2003; McGuire et al. 2007; Voros et al. 2006). However, if gene flow between species ends and sufficient time passes, the introgressed alleles may spread to other areas of the of the hybridizing species geographic range, obscuring the geographic area where hybridization occurred (Bossu and Near accepted; Ray et al. 2008).

As the geographic signal of introgression becomes less precise with time, determining the evolutionary timing of introgression events becomes easier, especially if relatively few alleles are introgressed. Calibrated molecular phylogenies offer a tool to estimate the absolute or relative ages of introgressed alleles within the receiving species (Hardman and Hardman 2008; Hollingsworth and Near in press; Near and Keck 2005; Near et al. 2005; Rabosky et al. 2007). However, using time-calibrated phylogenies for currently or recently hybridizing species where many alleles have introgressed will result in uninformative estimates of introgression frequency, because the credibility intervals of age estimates for the most recent common ancestor (MRCA) of the introgressed alleles will overlap and limit the ability to identify distinct introgression events.

Comparison of minimum genetic distances can provide chronological information when estimation methods would give ambiguous results. In this case, minimum genetic distances are the minimum observed distance between an introgressed haplotype and all haplotypes isolated from the donating species. Patterns in plots of minimum genetic distances between the introgressed haplotypes and those from the donating species can help to differentiate between episodic or continuous introgression. This is possible because no matter the distance between any of the originally introgressed haplotypes, they should diverge from the donating species' haplotypes at relatively the same rate, and thus will have similar minimum genetic distances from the donating species' haplotypes, assuming the haplotypes have been adequately sampled. For instance, if the introgression is episodic, the introgressed haplotypes should all have approximately the same minimum genetic distance, and plotting the numbers of haplotypes by the minimum genetic distances should result in a cluster around a given distance.

Specimen Collection, DNA Isolation and Sequencing

Fishes were collected using seines and/or a backpack electrofishing unit. Tissue biopsies were preserved in 100% ethanol, stored at 4^{o} C, and deposited into the Yale Fish Tissue Collection (YFTC). Voucher specimens were preserved by fixation in 10% formalin solution for at least seven days, washed in tap water for two days, and stored in 70% ethanol. Corresponding labels were stored with the tissue biopsies and attached to voucher specimens. Voucher specimens were deposited in either the University of Tennessee Research Collection of Fishes (UT) or the Yale Peabody Museum of Natural History fish collection (YPM). One non-darter percid and six non-*Nothonotus* darters were sampled as outgroup species (Table C.1). At least one individual of all other recognized *Nothonotus* was included (Table C.1). The three focus species, *N. camurus*, *N. chlorobranchius*, and *N. rufilineatus*, were collected from throughout their geographic ranges (Fig. 4.3 and Table C.1).

Nucleic acids were isolated using Qiagen DNAeasy kits according to the manufacturer's protocol. The mtDNA encoded cytochrome *b* (cyt*b*), nuclear encoded first intron of the S7 ribosomal protein, nuclear encoded RAG1 exon, and nuclear encoded MLL genes were amplified using primers and PCR protocols outlined in previous studies (Bossu and Near accepted). Successful PCR products were cleaned for sequencing using Qiagen Qiaquick PCR purification kits according to the manufacturer's protocol, or by digestion with 1.0 unit of both Exonuclease I and Shrimp Alkaline Phosphate and incubated for 15 min at 37^{o} C and 20 min at 80^{o} C, or by Polyethylene Glycol (PEG)

precipitation. In PEG an equal volume (usually ~23 ul) of 20% PEG solution was added to PCR products, this mixture was vortexed briefly and placed in a 37^{o} C incubation chamber for 15 min. After incubation it was placed in a centrifuge at ~6000 rcf for 20 min, the supernatant was removed, and the remaining DNA was washed with 200 ul of 70% ethanol. The ethanol was evaporated using a vacuum centrifuge for ~15 min and the DNA was resuspended in ~18 ul of H_2O. The prepared PCR product was used as a template and sequenced by the DNA Sequencing Facility on Science Hill at Yale University or WM Keck Foundation Biotechnology Resource Laboratory at Yale University. Contiguous sequences were assembled from individual sequence reaction files in Sequencher (Gene Codes, Ann Arbor, MI, USA). Sequences for cyt*b* and RAG1 were aligned by eye as there were no differences in sequence length between individuals. MUSCLE (Edgar 2004) or ClustalX (Thompson et al. 1997) was used to align S7 and MLL sequences. All sequences not used in previous studies (Keck and Near 2008; Near and Keck 2005) were deposited in GenBank with the accession numbers XXXXXX-XXXXXX (to be released when published). Identical sequences were removed using MacClade (D.R. Maddison and Maddison 2000) for certain analyses. For the concatenated dataset, individuals were removed only if all three genes were identical.

Genetic Analyses

Phylogenetic relationships were estimated using partitioned Bayesian analyses (Ronquist and Huelsenbeck 2003) with posterior probabilities estimated using metropolis-coupled Markov chain Monte Carlo (Huelsenbeck et al. 2001; Larget and Simon 1999) as executed in the computer program MrBayes 3.1 (Ronquist and Huelsenbeck 2003). The datasets were partitioned by codon position for coding sequences and as a single partition for non-coding sequences. The optimal maximum likelihood model of molecular evolution for each partition was determined using AIC as executed in the computer program Modeltest 3.0 (Posada and Crandall 1998). We ran seven separate analyses, one for each gene individually, two on subsets of the RAG1 dataset, and one on a concatenated dataset that included the three nuclear genes. Each phylogenetic analysis was run for 10 million generations in MrBayes 3.1 on the computer cluster at the Computational Biology Service Unit at Cornell University. The first 2 million generations were discarded as burn-in and the posterior probabilities of nodes were calculated from the remaining post burn-in trees.

Allele networks were created using median joining methods (Bandelt et al. 1999) in the software Network 4.5 (fluxus-engineering.com). Invariant sites were removed from the datasets in PAUP 4.0 (Swofford 2003) before running the analyses in Network 4.5. The datasets used in the network analyses contained sequences from all individuals, including identical haplotypes and alleles. One cyt*b* dataset included *N. camurus* (except *N. camurus* with *N. chlorobranchius* haplotypes) and *N. rufilineatus* with *N. camurus* haplotypes. The second cyt*b* dataset contained the *N. camurus* and *N. rufilineatus* with *N. chlorobranchius* haplotypes and *N. chlorobranchius*. The datasets for the nuclear genes contained all *Nothonotus* individuals.

Arlequin (Excoffier et al. 2005) was used for AMOVA between allopatric and sympatric populations of the focus species and populations of *N. ruflineatus*. Introgression of the nuclear genes would result in the amount of variation being higher

for populations within groups than among groups in sympatric populations than in the allopatric populations. Additionally, if *N. rufilineatus* is divided into populations delineated by the mito-type they harbor, AMOVA should show more variation explained among groups than populations within groups if there is introgression of the nuclear genes; however, this result could not be differentiated from structure arising form other processes. For these analyses, river systems within major drainages were considered populations. For the 'Global' accounting of variance across all three nuclear genes we summed the 'among groups', 'among populations within groups', 'within population', and 'total' variance components for each comparison and then calculated the percentages for each of the partitions.

A uniquely reduced dataset was used to find the minimum genetic distances for cyt*b* between introgressed haplotypes and the haplotypes observed in the donating species. The beginning dataset contained sequences from all individuals of *N. camurus*, *N. chlorobranchius*, and *N. rufilineatus* with the individuals containing introgressed haplotypes labeled as to the donating species. Identical haplotypes within species, as opposed to across all species in the phylogenetic analyses above, were removed to avoid over-representation of specific distances. If these remained in the analyses, the plots of number of haplotypes by distance could incorrectly support that there was an influx of introgressed haplotypes around the same time. Genetic distances were calculated using PAUP 4.0. The minimum genetic distance for each introgressed haplotype was identified from all distances between that haplotype and the donating species' haplotypes. The minimum genetic distances were rounded to 10^{-3} and the numbers of haplotypes per distance were plotted.

RESULTS

Localities, Sequences and Model Selection

Three hundred and seventeen individuals of the three focus species were sampled from over 70 different localities (Fig. 4.3 and Table C.1). Two hundred to over three hundred individuals were sequenced for each gene (Table 4.1). Removal of redundant alleles resulted in less than 50% reduction in the number of individuals in each gene dataset except for MLL, which was reduced by 68% (Table 4.1). Uncorrected genetic distances for cyt*b* sequences within and among the three focus species (with their 'own' mitochondrial haplotype) were congruent with results from previous studies that used cyt*b* sequences to investigate *Nothonotus* phylogeny (Keck and Near 2008; Near and Keck 2005) (Table 4.2). Genetic distances of individuals with captured mitochondrial haplotypes were within the ranges of genetic distances between haplotypes sampled from the donor species (Table 4.2). The maximum genetic distances between *N. rufilineatus* with captured haplotypes and the donating species were greater than the maximum genetic distances within the corresponding donating species (Table 4.2). Uncorrected genetic distances of the three nuclear genes for the three focal species and *N. rufilineatus* by population showed that there was more divergence in S7 than MLL or RAG1 (Table 4.3). The average genetic distance was higher in *N. rufilineatus* for all three nuclear genes than in the other two species (Table 4.3). Within *N. rufilineatus*, the lower Tennessee population had the highest average genetic distances for each nuclear gene and the Cumberland River population had substantially lower average and maximum genetic

Table 4.1. Number of individuals sequenced, unique haplotypes or alleles, and aligned sequence length by gene.

Gene	# Individuals	# Haplotypes or alleles	Sequence Length
cytb	317	197	1140 bp
MLL	206	65	720 bp
S7	274	181	550 bp
RAG1	221	112	1319 bp

Table 4.2. Genetic distances for cyt*b* given as percent values within and among different groups of focus species. *N. camurus* (Ncam), *N. camurus* with *chlorobranchius*-like haplotypes (Ncam w/chb), *N. chlorobranchius* (Nchb), *N. rufilineatus* (Nruf), *N. rufilineatus* with *camurus*-like haplotypes (Nruf w/cam), and *N. rufilineatus* with *chlorobranchius*-like haplotypes (Nruf w/chb). Top number is the minimum distance, middle number is the average distance, and the bottom number is the maximum distance.

%	Nchb	Ncam	Ncam w/chb	Nruf	Nruf w/chb	Nruf w/cam
	0.00					
Nchb	1.43					
	3.16					
	2.98	0.00				
Ncam	3.38	0.52				
	4.21	1.40				
	0.09	2.89	0.35			
Ncam	1.52	3.23	0.64			
w/chb	2.98	3.86	1.14			
	9.65	9.47	9.82	0.00		
Nruf	10.21	10.10	10.22	1.15		
	10.88	10.61	10.61	2.19		
	0.00	2.81	0.00	9.74	0.00	
Nruf	1.32	3.21	0.80	10.31	0.95	
w/chb	3.24	4.12	2.89	10.96	2.98	
	2.98	0.00	2.89	9.56	2.81	0.00
Nruf	3.40	0.59	3.25	10.05	3.22	0.46
w/cam	3.95	1.84	3.77	10.70	3.95	1.40

Table 4.3. Uncorrected genetic distances given as percentages for each gene by species and *N. rufilineatus* by population. Average genetic distance is the top number and maximum genetic distance is the lower number (minimum genetic distance was 0 for all groups). Genetic distances of *N. rufilineatus* cyt*b* are only given by populations delineated by mito-type.

	Gene			
Species	cyt*b*	S7	MLL	RAG1
N. camurus	0.52	0.54	0.02	0.05
	1.40	3.06	0.30	0.45
N. chlorobranchius	1.43	0.59	0.04	0.03
	3.16	1.73	1.41	0.15
N. rufilineatus	n/a	1.45	0.24	0.29
All	n/a	4.02	0.99	0.76
N. rufilineatus	0.95	1.18	0.16	0.21
upper Tennessee	2.98	3.82	0.56	0.68
N. rufilineatus	1.15	1.74	0.32	0.25
lower Tennessee	2.19	3.50	0.99	0.68
N. rufilineatus	0.46	0.42	0.07	0.05
Cumberland	1.40	1.34	0.28	0.15

Phylogenetic Inferences

Nothonotus was monophyletic in all phylogenetic analyses except in the Bayesian analysis of the nuclear encoded MLL gene tree that was largely unresolved. The Bayesian inferred cyt*b* gene tree was well resolved (Fig. 4.4) and was similar to previous studies of *Nothonotus* phylogeny (Keck and Near 2008; Near and Keck 2005). *Nothonotus rufilineatus* and *N. camurus* mitochondrial haplotypes were not monophyletic in the mitochondrial cyt*b* gene tree. *Nothonotus rufilineatus* mitochondrial haplotypes were distributed among three clades in the *Nothonotus* phylogeny (Fig. 4.4). There were 116 unique cyt*b* haplotypes observed among the 186 sampled *N. rufilineatus* specimens. A clade that contained 36 mitochondrial haplotypes observed in *N. rufilineatus* specimens sampled from the Duck River and Tennessee River tributaries below the Flint River was the sister lineage of a clade containing *N. tippecanoe* and *N. denoncourti* (Fig. 4.4). For the remainder of the discussion, the haplotypes contained in this clade are called *rufilineatus*-like haplotypes. The majority of mitochondrial haplotypes observed in *N. rufilineatus* specimens were found outside of this clade. All mitochondrial haplotypes observed in *N. rufilineatus* specimens sampled from the Tennessee River drainage above the Flint River were phylogenetically nested in a clade that contained all of the mitochondrial haplotypes sampled from *N. chlorobranchius* specimens, and the haplotypes contained in this clade will be referred to as *chlorobranchius*-like haplotypes. *Nothonotus rufilineatus* mtDNA haplotypes sampled from the Cumberland River drainage were nested in a clade that contained all sampled *N. camurus* specimens (Fig. 4.4) and the haplotypes contained in this clade will be referred to as *camurus*-like haplotypes. Seven of the mtDNA haplotypes observed in seven *N. camurus* individuals were *chlorobranchius*-like haplotypes. The relationships within the *N. camurus* and *N. chlorobranchius* clades in the cyt*b* gene tree were not resolved, and none of the three species represented in these clades formed monophyletic groups.

Bayesian analyses of the DNA sequences sampled from each of three nuclear genes resulted in varying levels of resolution, and the concatenated three nuclear gene dataset resulted in a phylogeny with the greatest resolution and proportion of nodes supported with significant Bayesian posterior probability values. The results of the Bayesian analysis of the S7 intron were similar to those reported in Keck and Near (2008), with very little phylogenetic resolution and few clades supported with significant Bayesian posterior probabilities (Fig. 4.5). The Bayesian inferred MLL gene tree had slightly greater resolution and posterior support (Fig. 4.6). However, *Nothonotus* was not monophyletic. *Nothonotus juliae* was sister to *Etheostoma vitreum*, but this node was not supported with a significant Bayesian posterior probability. Several clades were supported with significant Bayesian posterior probabilities including the *N. tippecanoe* and *N. denoncourti* clade and *N. jordani* clade that contains all of the *Nothonotus* species endemic to the Mobile Basin (Wood and Mayden 1993). Two egg-burying species, *N. moorei* and *N. rubrus*, were nested in the egg-guarding *N. maculatus* clade, a topology found in non-genetics based phylogenies (Etnier and Williams 1989). The MLL alleles sampled from *N. rufilineatus*, *N. camurus*, *N. chlorobranchius*, and *N. bellus* were clustered in a clade that was not supported with a significant Bayesian posterior probability (Fig. 4.6). The Bayesian phylogenetic analysis of the RAG1 locus, full dataset resulted in phylogeny with higher resolution when compared to the phylogenies inferred

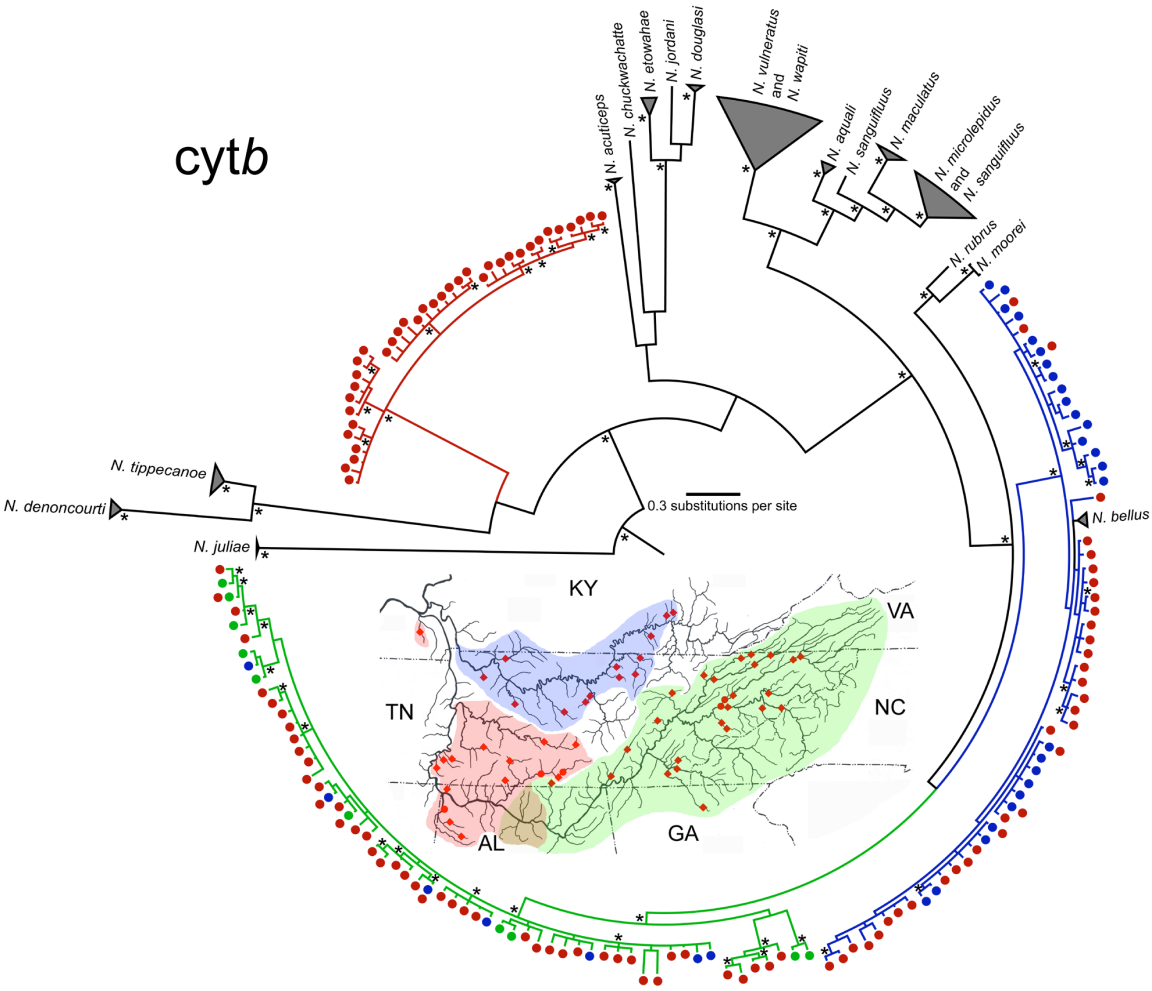

Fig. 4.4. Phylogeny resulting from Bayesian analysis of the mitochondrial cytochrome *b* dataset. All nodes supported with a Bayesian posterior probability of > 0.94 are indicated with asterisks. Red dots indicate *N. rufilineatus* haplotypes and red branches indicate the *N. rufilineatus* clade. Blue branches indicate the *N. camurus* clade and blue dots indicate haplotypes isolated from *N. camurus* individuals. Green branches indicated the *N. chlorobranchius* clade and green dots indicate haplotypes isolated from *N. chlorobranchius* individuals. A haplotype shared between species is indicated with multiple dots of different colors at a terminus. Inside of the phylogeny is a map of the Cumberland and Tennessee Rivers showing the distribution of mitochondrial haplotypes in *N. rufilineatus*. Red markers indicate sampled localities. Red shading indicates the range of *rufilineatus*-like haplotypes sampled from *N. rufilineatus*. Blue shading indicates the range of *camurus*-like haplotypes sampled from *N. rufilineatus*. Green shading indicates the range of *chlorobranchius*-like haplotypes sampled from *N. rufilineatus*.

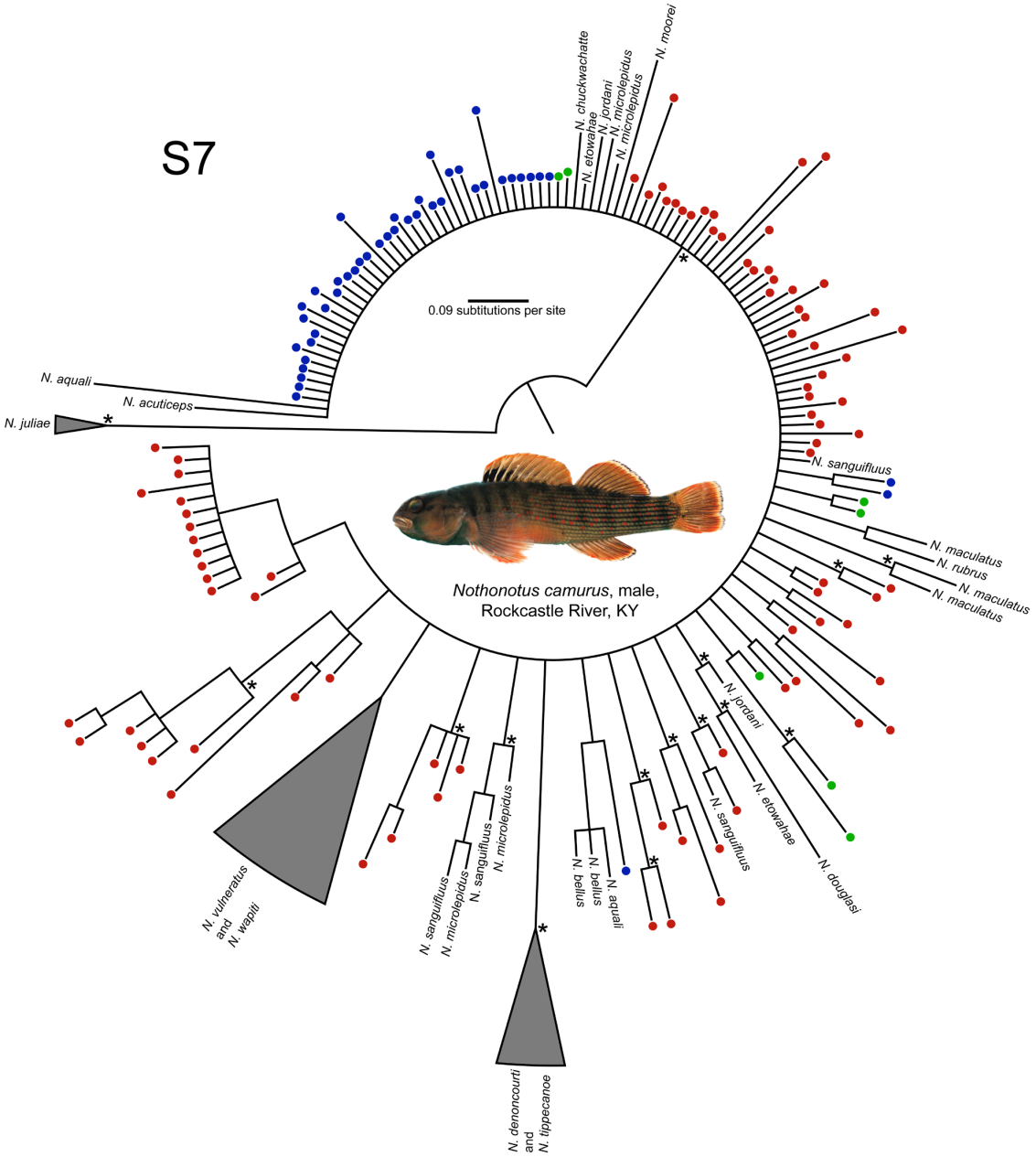

Fig. 4.5. Phylogeny resulting from Bayesian analysis of the nuclear encoded S7 ribosomal intron dataset. All nodes supported with a Bayesian posterior probability of > 0.94 are indicated with asterisks. Blue dots indicate alleles isolated from *N. camurus* individuals, green dots indicate alleles isolated from *N. chlorobranchius* individuals, and red dots indicate alleles isolated from *N. rufilineatus* individuals.

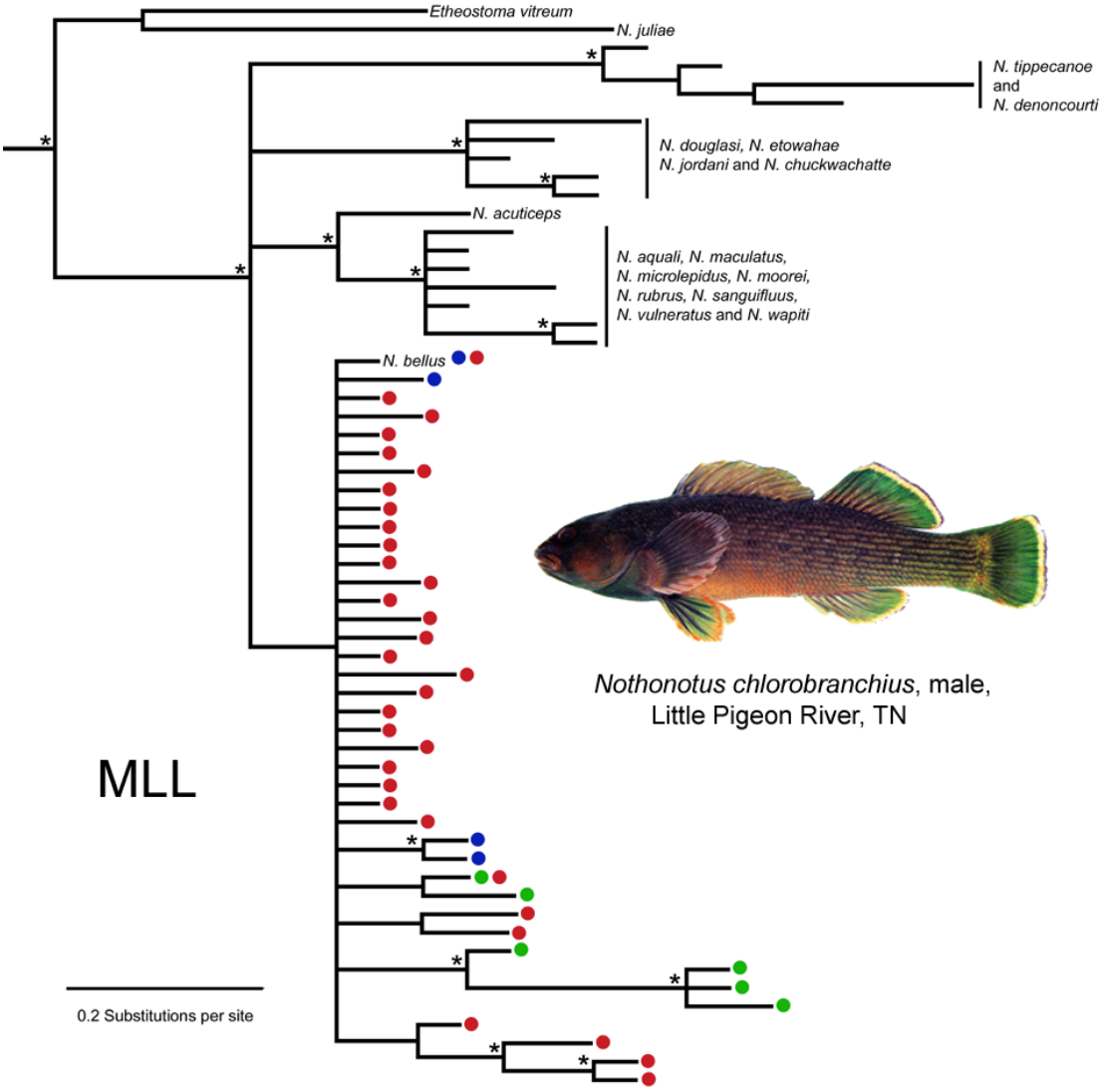

Fig. 4.6. Phylogeny resulting from Bayesian analysis of the nuclear encoded MLL exon dataset. All nodes supported with a Bayesian posterior probability of > 0.94 are indicated with asterisks. Blue dots indicate alleles isolated from *N. camurus* individuals, green dots indicate alleles isolated from *N. chlorobranchius* individuals, and red dots indicate alleles isolated from *N. rufilineatus* individuals.

from the S7 intron and MLL nuclear genes (Figs. 4.5 and 4.6). Alleles observed in *N. chlorobranchius* and *N. rufilineatus* were each monophyletic and supported with significant Bayesian posterior probabilities, but the alleles observed in *N. camurus* were not monophyletic (Fig. 4.7). The Bayesian analyses of the reduced *N. ruflineatus* RAG1 datasets resulted in phylogenies identical to the full dataset, including a monophyletic *N. rufilineatus*, with all nodes and significant support the same other than those missing because of removed alleles (Figs. C.1 and C.2). The Bayesian analysis of the concatenated dataset resulted in a phylogeny that, relative to the gene trees estimated from each of the nuclear genes, was resolved and supported with Bayesian posterior probabilities (Fig. 4.8). Sampled specimens of *N. chlorobranchius* and *N. rufilineatus* were each monophyletic with significant Bayesian posterior probabilities. *Nothonotus chlorobranchius* and *N. bellus* alleles were nested within a paraphyletic grouping of sampled *N. camurus* specimens.

Networks

The network resulting from analysis of the mitochondrial cyt*b* clade of *camurus*-like haplotypes exhibited geographic structure (Fig. 4.9). Partition A contained *camurus*-like haplotypes observed in *N. camurus* specimens sampled from the upper Tennessee River drainage and the Wabash, Kentucky, Scioto, Muskingum, Elk (West Virginia), and Allegheny river systems, and *camurus*-like haplotypes observed in a few of the *N. rufilineatus* specimens sampled from the upper Tennessee River drainage. Partition B contained the *camurus*-like haplotypes observed in *N. camurus* and all *N. rufilineatus* specimens sampled from the Cumberland River drainage. The minimum spanning network of the mitochondrial *chlorobranchius*-like haplotypes contained the haplotypes observed in all sampled *N. chlorobranchius* specimens, the haplotypes observed in most of the sampled *N. rufilineatus* specimens sampled from the upper Tennessee River drainage, and the haplotypes observed in a few of the sampled *N. camurus* specimens (Fig. 4.10). The *chlorobranchius*-like haplotypes observed in *N. rufilineatus* and *N. chlorobranchius* sampled from the Taccoa, Little Tennessee, and Hiwassee + Ocoee river systems clustered geographically, but the remaining haplotypes did not show a strong geographic correlation. Six of the seven *chlorobranchius*-like haplotypes found in *N. camurus* were more similar to *chlorobranchius*-like haplotypes observed in *N. rufilineatus* than they were to *chlorobranchius*-like haplotypes found in *N. chlorobranchius*. The one *N. camurus* with a *chlorobranchius*-like haplotype that was more similar to *N. chlorobranchius* was from the Nolichucky River.

Allele networks for MLL and RAG1 had some structure between *Nothonotus* species, but little geographic signal within the focal species (Figs. 4.11 and 4.12). The network for S7 had little structure with many connections for each allele and is not shown. In the MLL network there is no geographic structure within the focal species and *N. rufilineatus* shares the most common allele with *N. camurus* (Fig. 4.11). In the RAG1 network there was no geographic structure within *N. camurus* or *N. chlorobranchius*, but there were three clusters in the *N. ruflineatus* alleles (Fig. 4.12). The RAG1 alleles from Cumberland River drainage *N. rufilineatus* clustered together and were most similar to an allele sampled from both the upper and lower Tennessee River populations of *N. rufilineatus* (Fig. 4.12). *Nothonotus rufilineatus* RAG1 alleles from Horse, Hardin, and

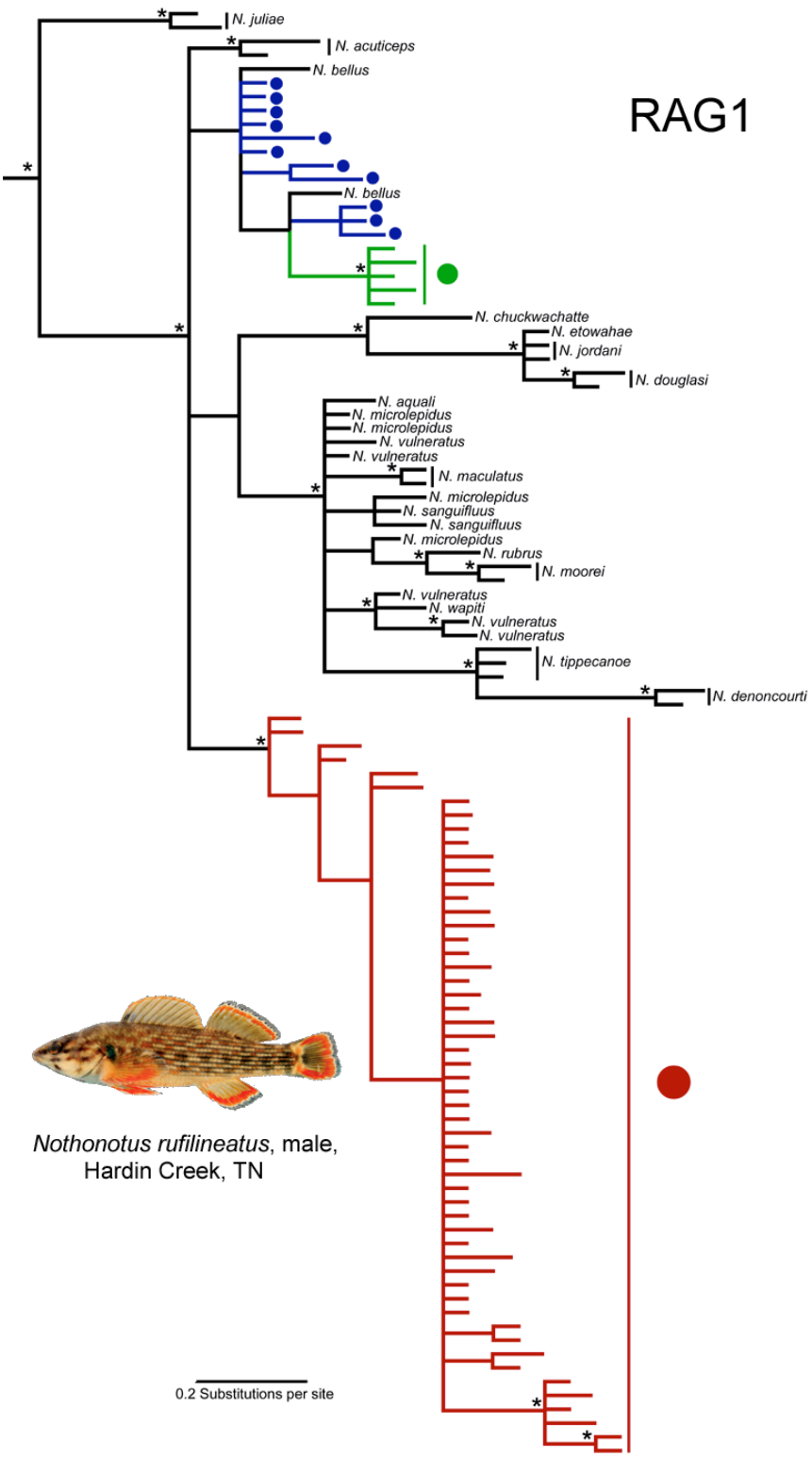

Fig. 4.7. Phylogeny resulting from Bayesian analysis of the RAG1 exon dataset. All nodes supported with a Bayesian posterior probability of > 0.94 are indicated with asterisks. Blue dots indicate alleles isolated from *N. camurus* individuals, green dots indicate alleles isolated from *N. chlorobranchius* individuals, and red dots indicate alleles isolated from *N. rufilineatus* individuals.

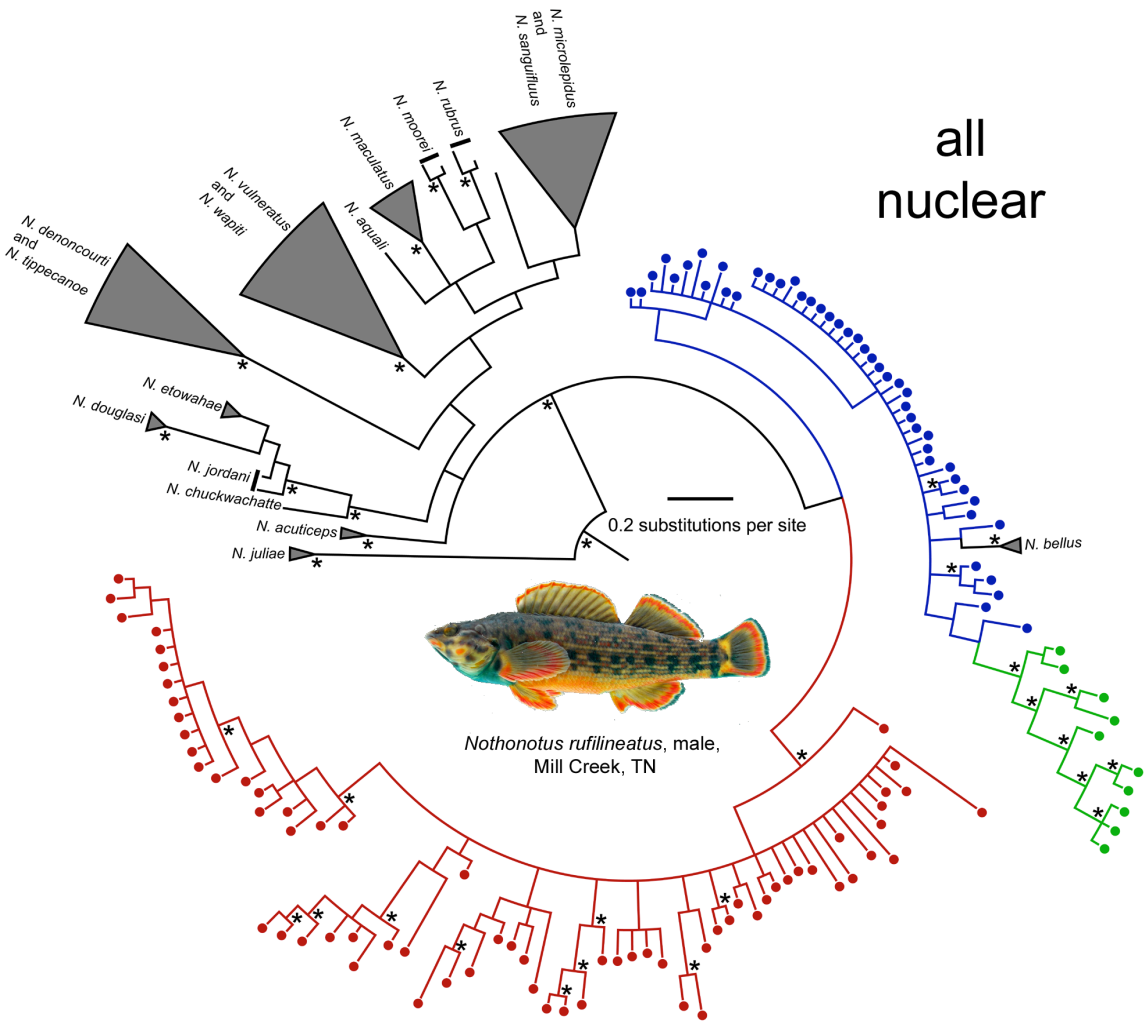

Fig. 4.8. Phylogeny resulting from Bayesian analysis of the concatenated nuclear loci dataset. All nodes supported with a Bayesian posterior probability of > 0.94 are indicated with asterisks. Blue dots indicate alleles isolated from *N. camurus* individuals, green dots indicate alleles isolated from *N. chlorobranchius* individuals, and red dots indicate alleles isolated from *N. rufilineatus* individuals.

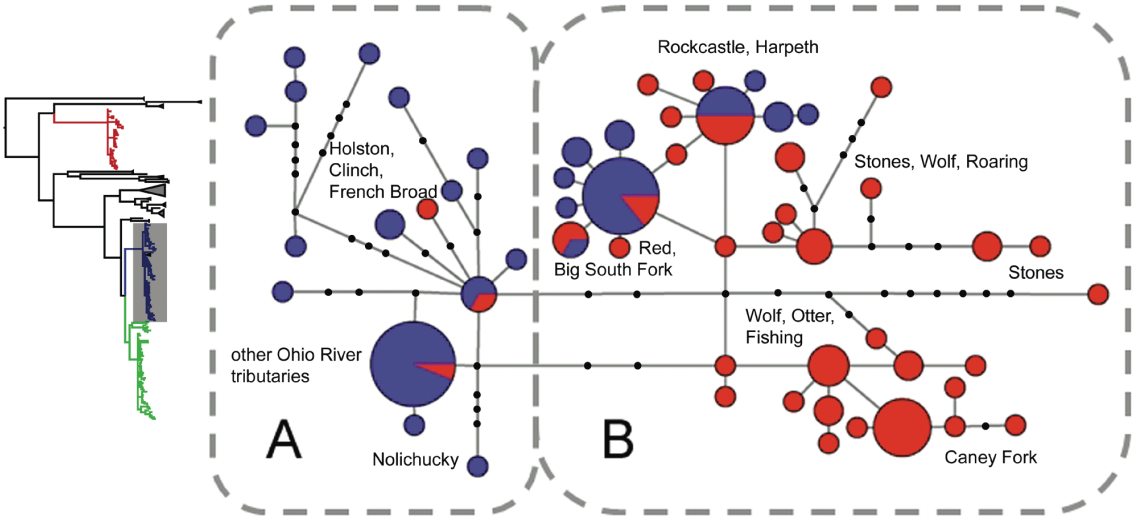

Fig. 4.9. Network resulting from median joining of *camurus*-like haplotypes sequenced from *N. camurus* and *N. rufilineatus*. The cyt*b* tree is on the left with a shaded area indicating the portion of the tree from which the haplotypes in the network are found. Blue indicates haplotypes isolated from *N. camurus* and red indicates haplotypes isolated from *N. rufilineatus*. Circle diameter is positively associated with number of individuals with that haplotype. Black dots on branches indicate base pair changes not represented in observed haplotypes. The portion of the network in the **A** partition are from the Tennessee River drainage and all Ohio River tributaries. The portion of the network in the **B** partition are from the Cumberland River drainage. Originating river systems for clusters of haplotypes are given near the clusters.

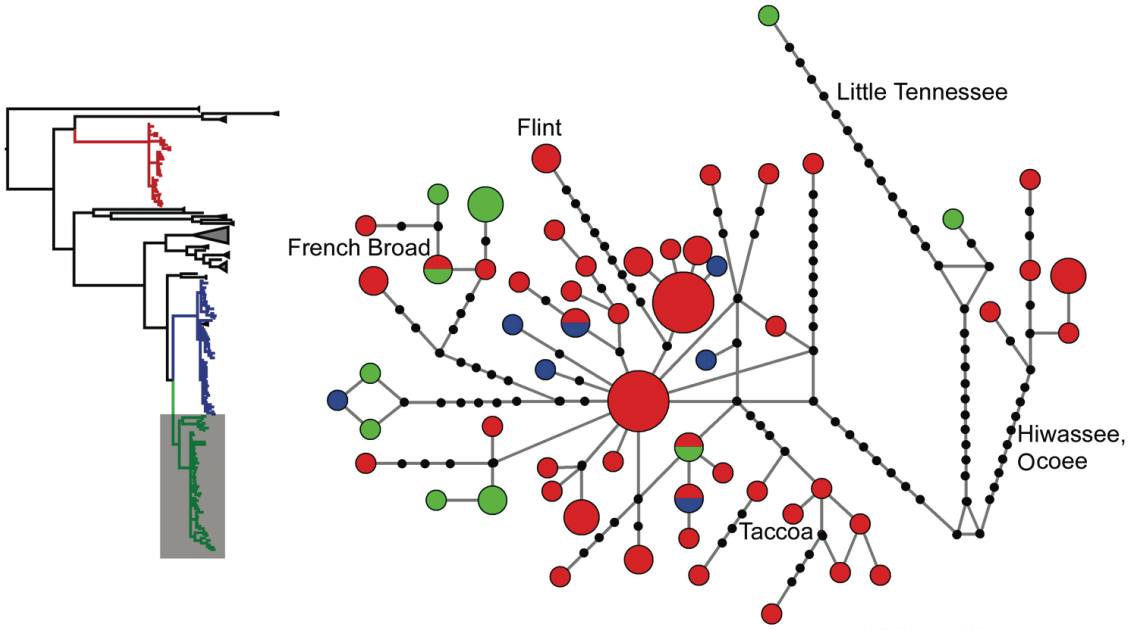

Fig. 4.10. Network resulting from median joining of *chlorobranchius*-like haplotypes sequenced from *N. chlorobranchius*, *N. camurus*, and *N. rufilineatus*. The cyt*b* tree is on the left with a shaded area indicating the portion of the tree from which the haplotypes in the network are found. Green indicates haplotypes isolated from *N. chlorobranchius*, blue indicates haplotypes sequenced from *N. camurus*, and red indicates haplotypes isolated from *N. rufilineatus*. Circle diameter is positively associated with number of individuals with that haplotype. Black dots on branches indicate base pair changes not represented in observed haplotypes. Originating river systems for clusters of haplotypes are given near the clusters, although not all are identified.

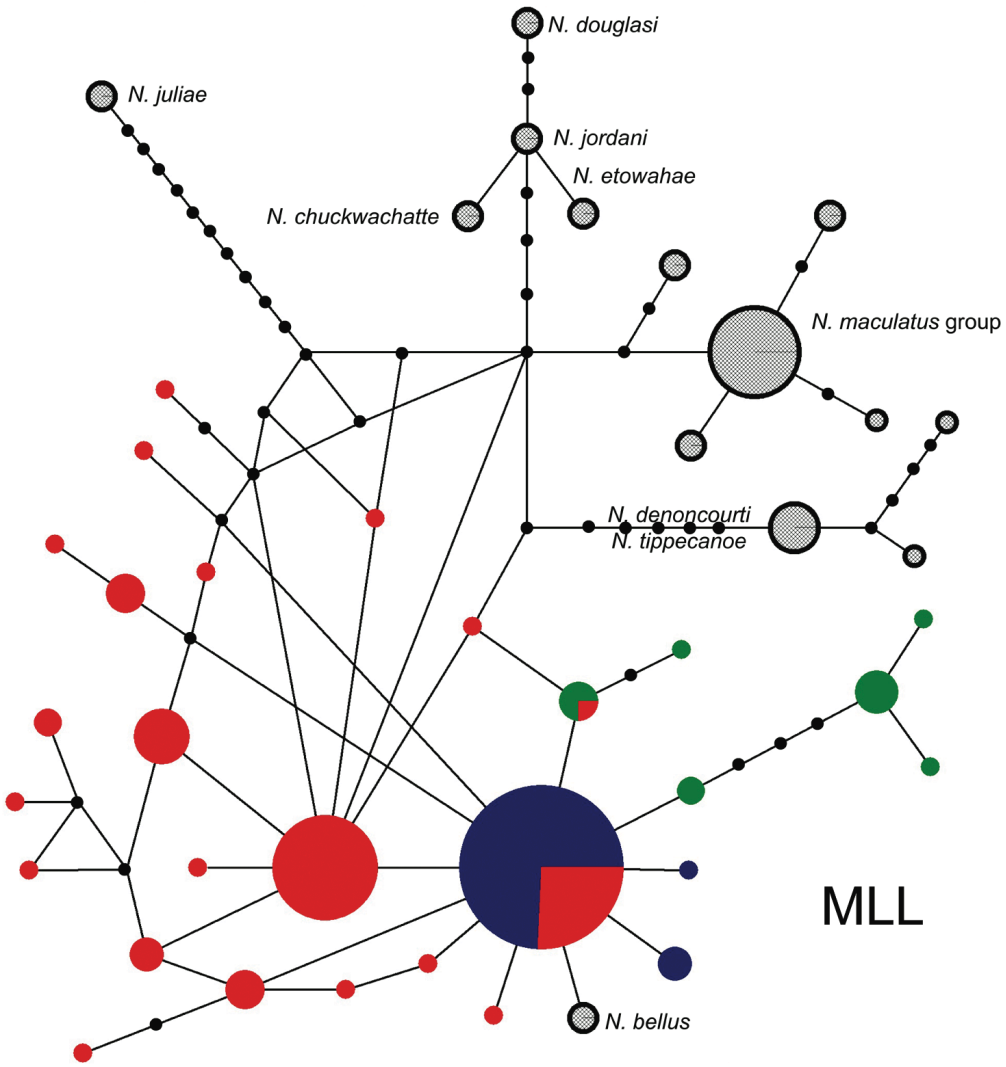

Fig. 4.11. Network resulting from median joining of all *Nothonotus* MLL alleles. Green indicates alleles isolated from *N. chlorobranchius*, blue indicates alleles isolated from *N. camurus*, red indicates alleles isolated from *N. rufilineatus*, and gray indicates other *Nothonotus* species. Circle diameter is positively associated with number of individuals with that allele. Black dots on branches indicate alleles not observed.

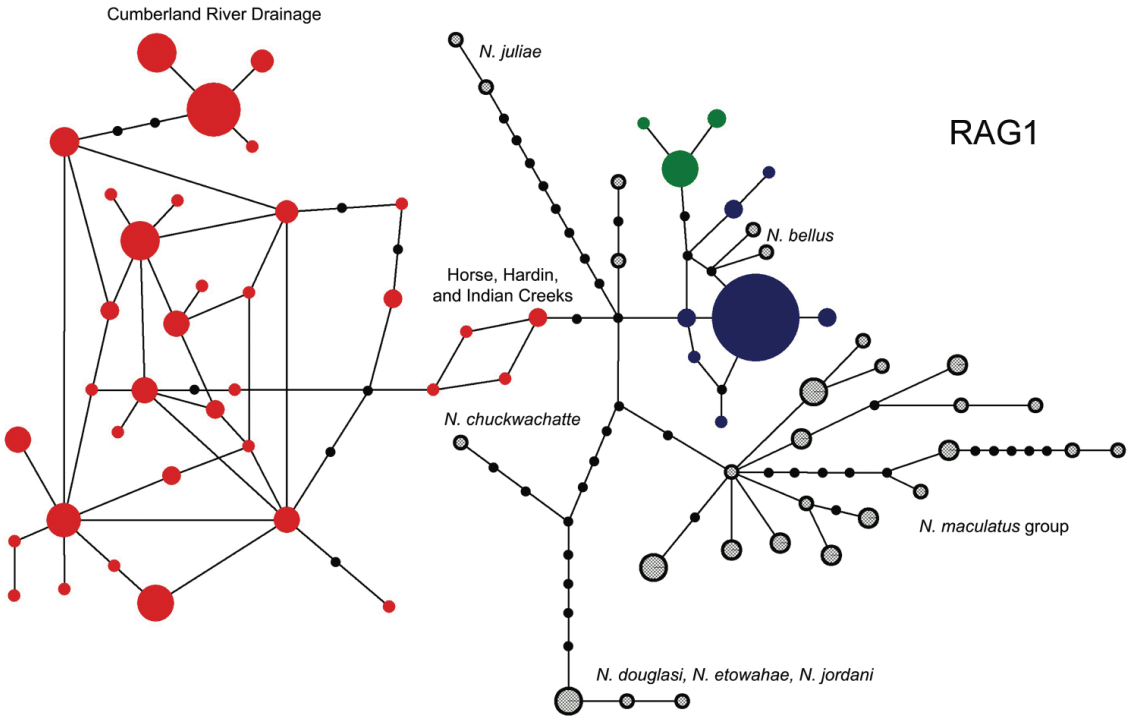

Fig. 4.12. Network resulting from median joining of all *Nothonotus* RAG1 alleles. Green indicates alleles isolated from *N. chlorobranchius*, blue indicates alleles isolated from *N. camurus*, red indicates alleles isolated from *N. rufilineatus*, and gray indicates other *Nothonotus* species. Circle diameter is positively associated with number of individuals with that allele. Black dots on branches indicate alleles not observed. Originating river systems for clusters of alleles are given near the clusters.

Indian Creeks, tributaries in the lower Tennessee River, clustered together and were nearly as similar to alleles from *N. camurus* and *N. acuticeps* as they were to the next most similar *N. rufilineatus* allele (Fig. 4.12). The remaining *N. rufilineatus* RAG1 alleles had no discernable structure.

AMOVA

All but three variance components were significant with p-values < 0.003 and the percentage of variance among groups across all three genes (global) was highest in all comparisons except between *N. rufilineatus* from the upper vs. lower Tennessee River drainages (Table 4.4). The among group variance was higher, for the genes individually and globally, in the comparison of *N. rufilineatus* from the Cumberland River drainage to *N. camurus* from the Cumberland River drainage than the among group variance in the comparison of *N. rufilineatus* from the lower Tennessee River drainage to *N. camurus* from the Cumberland River drainage (Table 4.4). The among group variance was higher, for MLL, RAG1, and globally, in the comparison of *N. rufilineatus* from the upper Tennessee River drainage to *N. chlorobranchius* than the among group variance in the comparison of *N. rufilineatus* from the lower Tennessee River drainage to *N. chlorobranchius* (Table 4.4). In the comparison of all *N. rufilineatus* and all *N. camurus*, variance in S7 and MLL was fairly equally distributed across components, but more variation was accounted for among groups in RAG1 and Globally (Table 4.4). In the comparison of all *N. rufilineatus* and all *N. chlorobranchius*, variance in S7 was fairly equally distributed across components, but more variation was accounted for among groups in MLL, RAG1, and Globally (Table 4.4). In the comparison of Cumberland River drainage and lower Tennessee River drainage *N. rufilineatus*, most of the variation was accounted for among groups in RAG1 and Globally, but less so in S7 and within populations accounted for most of the variation in MLL (Table 4.4). In the comparison of upper Tennessee River drainage and lower Tennessee River drainage *N. rufilineatus*, most of the variation was accounted for within populations and the least variation was accounted for by among groups in all genes and Globally (Table 4.4).

Minimum Distances

The plots of number of haplotypes per minimum uncorrected genetic distance were different for each group of hypothesized captured mitochondrial haplotypes (Fig. 4.13). The minimum distances for *camurus*-like mitochondrial haplotypes observed in *N. rufilineatus* were all less than 1.0% divergent from *camurus*-like haplotypes observed in *N. camurus*, and most of these contrasts were less than 0.5% (Fig. 4.13a). Two clusters of minimum distances were evident in the plot of genetic distances of *chlorobranchius*-like haplotypes observed in *N. rufilineatus* specimens sampled from the upper Tennessee River drainage with *chlorobranchius*-like haplotypes observed in *N. chlorobranchius* specimens (Fig. 4.13b). Most of the genetic distances were distributed near 0.5% and a second cluster of genetic distances was observed 2.0 and 2.5%. All the minimum genetic distances between the *chlorobranchius*-like mitochondrial haplotypes observed in *N. camurus* with *chlorobranchius*-like haplotypes observed in *N. chlorobranchius* were all less than 0.5%, but none of the contrasted haplotypes were identical (Fig. 4.13c). The minimum distances between *chlorobranchius*-like mitochondrial haplotypes observed in

Table 4.4. Results of AMOVA for each gene between groups of *N. camurus* (Ncam), *N. chlorobranchius* (Nchb), and *N. rufilineatus* (Nruf). Given as percentages of variation accounted for among groups (top number), among populations within groups (middle number), and within populations (bottom number). The Global numbers were calculated from the sums of the variance components across the three genes as described in the methods section. Groups are identified as being from the Cumberland River drainage (Cumberland), lower Tennessee River drainage (lower Tennessee), upper Tennessee River drainage (upper Tennessee), Ohio River tributaries other than the Tennessee and Cumberland River drainages (Ohio trib.), and all populations of a species (All). All Variance Components were significant with p-values < 0.003 unless noted: * < 0.01 and ** > 0.01.

Populations	S7	MLL	RAG1	Global
Nruf Cumberland	81.77	63.14	92.79	85.80
and	11.01	24.73	2.99	8.08
Ncam Cumberland	7.22	7.22	4.23	6.12
Nruf lower Tennessee	41.53	33.41	68.00	48.84
and	31.67	28.42	16.29	26.67
Ncam Cumberland	26.80	38.17	15.72	24.49
Nruf Cumberland	78.69	64.09	94.51	85.13
and	11.16	22.05	1.84	7.47
Ncam Ohio trib.	10.15	13.85	3.65	7.39
Nruf All	35.83	25.36	68.97	48.12
and	38.26	37.80	18.85	30.61
Ncam All	25.91	36.84	12.18	21.26
Nruf upper Tennessee	29.74	47.43	84.57	55.53
and	27.14	27.88	0.71**	16.33
Nchb All	43.12	24.69	14.73	28.14
Nruf lower Tennessee	34.54	45.34	74.22	48.71
and	35.44	29.19	13.50	27.55
Nchb All	30.02	25.47	12.29	23.73
Nruf All	32.60	52.46	69.28	49.49
and	40.82	25.66	19.88	30.61
Nchb All	26.58	21.88	10.84	19.90
Ncam All	51.29	70.43	86.17	64.42
and	26.86	17.80	2.43**	18.47
Nchb All	21.86	11.77	11.40	17.10
Nruf Cumberland	38.76	20.77	66.71	45.77
and	32.15	31.29	16.62	27.19
Nruf lower Tennessee	29.09	47.94	16.67	27.04
Nruf upper Tennessee	11.58	23.06	17.17	14.36
and	38.66	26.51	25.81	34.44
Nruf lower Tennessee	49.77	50.43*	57.02	51.20

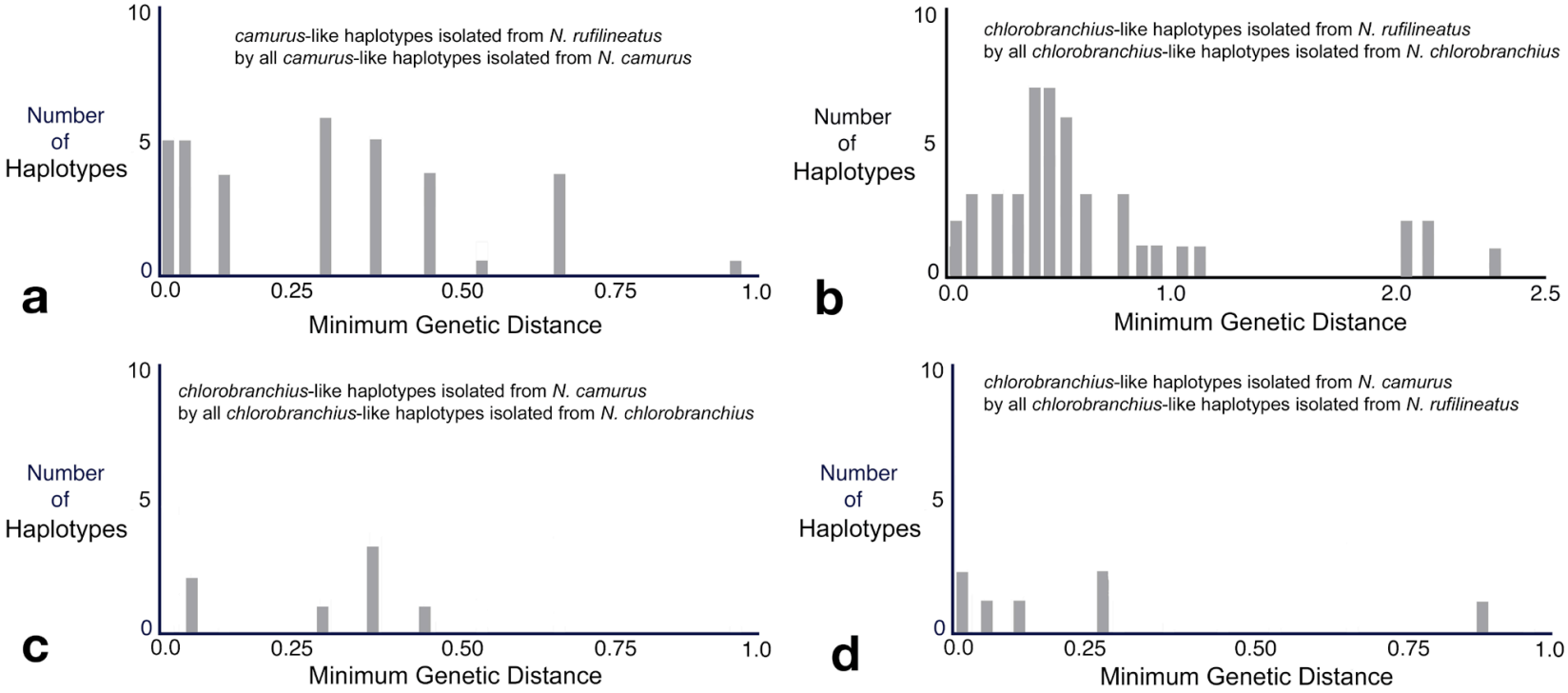

Fig. 4.13. Graphs indicating the number of haplotypes per minimum genetic distance.

N. camurus with *chlorobranchius*-like haplotypes observed in *N. rufilineatus* ranged between 0% to just less than 1.0% (Fig. 4.13d).

DISCUSSION

The genetic diversity of *N. rufilineatus* was noticeably different both between populations of *N. rufilineatus* and between it and the other two species. The Cumberland River *N. rufilineatus* had substantially fewer alleles for each of the three nuclear genes than the other populations of *N. rufilineatus* and this is consistent with a population bottleneck in the past. The reduced diversity in this population inflated the among groups component in the AMOVA results in the comparisons containing this group and this was considered in making the inferences in the discussion. The significant Bayesian posterior supporting a monophyletic *N. rufilineatus* in the analysis of the RAG1 dataset (Fig. 4.7) could have been affected by a bias in parameter optimization towards the substantially larger number of unique *N. rufilineatus* alleles than from the other two species. However, we found the same support and relationships in the trees resulting from datasets with reduced *N. rufilineatus* allele numbers, indicating there was no effect of bias (Figs. C.1 and C.2).

Introgression?

The clustering of mitochondrial haplotypes isolated from *N. rufilineatus* in three distinct regions of the *Nothonotus* phylogeny is inconsistent with previous phylogenies based on morphological, behavioral, allozyme, and limited genetic data (Etnier and Williams 1989; Keck and Near 2008; Near and Keck 2005; Wood 1996) and may be the result of: 1) undescribed diversity due to a polytypic *N. rufilineatus*, 2) stochastic sorting of ancestral polymorphism resulting in retention of polymorphism in *N. rufilineatus*, 3) introgression from an unknown source into the lower Tennessee River population of *N. rufilineatus*, if *N. rufilineatus'* true relationship is in a clade with *N. camurus* and *N. chlorobranchius*, or 4) introgression from *N. camurus* and *N. chlorobranchius* into Cumberland River drainage and upper Tennessee River drainage populations of *N. rufilineatus*, respectively, if *N. rufilineatus'* true position in the *Nothonotus* phylogeny is consistent with traditional placement. Including more nuclear genetic information and analyzing all the data from both phylogenetic and population biology perspectives supports the fourth scenario more than the others, but does not preclude considering these possibilities in future studies.

A polytypic *N. rufilineatus* is the least supported possibility, both genetically and morphologically. If the cyt*b* tree (Fig. 4.4) represents the species tree, then *N. rufilineatus* is polytypic, with one lineage in the traditional (Etnier and Williams 1989; Keck and Near 2008; Near and Keck 2005; Wood 1996) *N. rufilineatus* position, one lineage sharing haplotypes with *N. chlorobranchius*, and one lineage sharing haplotypes with *N. camurus*. Zorach (1970) found that *N. rufilineatus* morphology varied by river systems within drainages and concluded that no populations warranted subspecific designation, moreover, the variation he found does not correspond to the *N. rufilineatus* groups delineated by the three mitochondrial types. The nuclear RAG1 geneology shows a monophyletic *N. rufilineatus* with significant Bayesian support (Fig. 4.7) and this is unexpected given the faster sorting of mitochondria due to its one-quarter effective

population size (Avise et al. 1988; Moore 1995). The results of the AMOVAs are also inconsistent with some *N. rufilineatus* being more closely related to *N. camurus* and *N. chlorobranchius*, because the variation accounted for among groups is almost always higher for comparisons of *N. rufilineatus* populations and the two other species than in comparisons of *N. rufilineatus* populations with each other (Table 4.4).

Retention of ancestral polymorphism in *N. rufilineatus* cannot be ruled out, but the monophyly of *N. rufilineatus* RAG1 alleles (Fig. 4.7) and the fact that there are known hybrid specimens involving these three species (Chapter 2) support a hypothesis of introgression. The AMOVA results and nuclear genealogies indicate that there may be limited nuclear introgression for the genes considered here. The three focus species only share nuclear alleles for MLL (Fig. 4.6 and 4.11), but the fact that some MLL alleles are shared among five or more species indicate the sharing results from retained ancestral polymorphism and not introgression. Additionally, the among groups component in the AMOVAs comparing sympatric and allopatric populations of the three focal species was higher for almost all genes and globally in the sympatric populations (Table 4.7). If these nuclear genes were introgressing at the same rate as the mitochondria the sympatric populations should have much lower percentages for the among groups component. The traditional placement of *N. rufilineatus* (Etnier and Williams 1989; Keck and Near 2008; Near and Keck 2005; Wood 1996) and occurrence of hybrid specimens, including *N. rufilineatus* X *N. camurus* and *N. rufilineatus* X *N. chlorobranchius* (Chapter 2), supports a hypothesis of introgression from *N. camurus* and *N. chlorobranchius* into *N. rufilineatus* over a hypothesis of introgression from an unknown source into the lower Tennessee River *N. rufilineatus*.

Geography and Timing of Introgression

We were able to delineate the geographic and temporal aspects of mitochondrial replacement in *Nothonotus* darters using phylogenies, haplotype and allele networks, and minimum genetic distances. The cyt*b* geneology (Fig. 4.4) shows that *N. rufilineatus* with introgressed haplotypes are found in two general regions, the Cumberland River drainage and upper Tennessee River drainage, and the haplotype networks (Figs. 4.9 and 4.10) provide better resolution between river systems within those drainages. Patterns in the haplotype networks and minimum genetic distances indicate that introgression has occurred both in the past and contemporaneously (Figs. 4.9, 4.10, and 4.13). In addition, *N. rufilineatus* is acting as a 'conduit species', where it enables transfer of *chlorobranchius*-like mitochondrial haplotypes into *N. camurus*.

Every individual of *N. rufilineatus* from the Cumberland River drainage had a *camurus*-like mitochondrial haplotype and the cyt*b* tree shows haplotype sharing between *N. camurus* and *N. rufilineatus* as well as clades of haplotypes found only in one species (Fig. 4.4). Identical *camurus*-like haplotypes observed in both *N. camurus* and *N. rufilineatus* from the same locality or tributary indicate contemporaneous mitochondrial replacement in the Cumberland River drainage. The clades that only have *camurus*-like haplotypes observed in *N. rufilineatus* indicate past introgression and subsequent sorting and divergence (Carson and Dowling 2006). However, there was little significant support or resolution within the *camurus*-like haplotype clade, limiting inferences about geographic and temporal aspects of introgression in the Cumberland River drainage.

The results from the haplotype network (Fig. 4.9 partition B) and minimum genetic distance analysis (Fig. 4.13a) confirm the pattern in the cyt*b* tree for the haplotypes sampled from the Cumberland River drainage. The fairly equal dispersion in the minimum genetic distance plot indicates a regular rate of introgression and the haplotypes are mostly clustered by drainage in the haplotype network. A few *N. camurus* and *N. rufilineatus* from the Red River system share identical *camurus*-like haplotypes indicating contemporary introgression in this river, but the other shared *camurus*-like haplotype is observed in *N. rufilineatus* from the Harpeth River system and *N. camurus* from the Rockcastle River system. *Nothonotus rufilineatus* and *N. camurus* are not sympatric in these rivers, eliminating the possibility of contemporary introgression within these rivers. The more divergent *camurus*-like haplotypes observed in *N. rufilineatus* were from populations not sympatric with *N. camurus*, other than the Harpeth population, and this is consistent with a mitochondrial conveyor belt model (McGuire et al. 2007), where introgressed mitochondrial haplotypes spread through a population from a point and are more divergent the greater the distance from that source. However, *N. camurus* has been extirpated from the Stones River, Caney Fork River, and Cumberland River proper (Etnier and Starnes 1993; Zorach 1972), and because we cannot genetically sample these populations we cannot differentiate between *camurus*-like haplotypes in these populations originating from introgression within the river systems or entering through a conveyor belt type scenario.

The vast majority of *N. rufilineatus* from the middle to upper Tennessee River had *chlorobranchius*-like mitochondrial haplotypes and the cyt*b* tree (Fig. 4.4) provided some indications as to the timing and geography of the introgression. Haplotype sharing between *N. chlorobranchius* and *N. rufilineatus* and haplotypes exclusive to one species indicate that introgression has occurred both in the past and contemporaneously. The subclades of *chlorobranchius*-like haplotypes were mostly composed of haplotypes observed in individuals from the same river system, including haplotypes that were not exclusive to one species indicating that introgression has occurred in those river systems (Nolichucky River, French Broad River, Little Pigeon River). However, lack of resolution in the cyt*b* tree prevents identification of between river system relationships.

The minimum genetic distance plot (Fig. 4.13b) and the haplotype network (Fig. 4.10) have more resolution and indicate two temporally separated introgression events and a positive relationship between genetic distance and geographic distance from the areas where haplotypes are currently shared. The minimum genetic distance plot for *chlorobranchius*-like haplotypes (Fig. 4.13b) had two distinct clusters of distances, the first was between 0 and just over 1% and the second with distances over 2%. The breadth of the first is similar to the distances seen in the *camurus*-like haplotypes and is consistent with introgression contemporaneously or in the recent past with subsequent divergence and the second cluster could be a result of a much older introgression. The *chlorobranchius*-like haplotypes observed in *N. rufilineatus* from the younger cluster in the minimum genetic distance plot are more divergent in the most distant downstream and one of the more distant upstream drainages than are haplotypes from rivers nearer the French Broad system where haplotypes are shared, and is a pattern consistent with the spread of haplotypes away from the introgression source (Carson and Dowling 2006; McGuire et al. 2007). The *chlorobranchius*-like haplotypes from the second, older cluster

are from *N. rufilineatus* in the Hiwassee and Ocoee Rivers, and this population of *N. rufilineatus* has been hypothesized to be a distinct species (Zorach 1970). In his study, Zorach (1970) described the geographic pattern of a unique male color pattern in the Hiwassee as being most prevalent in the Hiwassee proper and less so in the Taccoa, where the majority of males were patterned like typical *N. rufilineatus*. We see the same division in the haplotype network (Fig. 4.10), with Hiwassee haplotypes being fairly distinct from the Taccoa haplotypes and the latter being more similar to *chlorobranchius*-like haplotypes from other Tennessee River drainage *N. rufilineatus*. The genetic and morphological evidence is consistent with Hiwassee *N. rufilineatus* being isolated and indicate that the introgressed *chlorobranchius*-like haplotypes are from an older introgression event. The majority of the upper Tennessee *N. rufilineatus* have *chlorobranchius*-like haplotypes that are more recently introgressed than the *chlorobranchius*-like haplotypes that are introgressed into Hiwassee *N. rufilineatus*.

Introgression in the upper Tennessee River drainage between *N. camurus* and the other two species is relatively infrequent, contemporaneous, and more frequent between *N. camurus* and *N. rufilineatus* than between *N. camurus* and *N. chlorobranchius*. Most notable is that *N. rufilineatus* is acting as a conduit species by transferring *chlorobranchius*-like mitochondrial genomes to *N. camurus*, where *chlorobranchius*-like mitochondrial haplotypes residing in *N. rufilineatus* are introgressed into *N. camurus* when these two species hybridize. In the cyt*b* tree (Fig. 4.4) and haplotype network (Fig. 4.9 partition A) two of the three *camurus*-like haplotypes observed in upper Tennessee *N. rufilineatus* are also found in *N. camurus*, indicating recent introgression from *N. camurus* into *N. rufilineatus*. Six of the seven *chlorobranchius*-like haplotypes observed in *N. camurus* were more similar to *chlorobranchius*-like haplotypes observed in *N. rufilineatus* than those observed in *N. chlorobranchius*, indicating they introgressed more recently from *N. rufilineatus* and not *N. chlorobranchius* (Fig. 4.4 and 4.10). All but one of the minimum genetic distances (Fig. 4.13c and d) show more similarity to *chlorobranchius*-like haplotypes in *N. rufilineatus* than *chlorobranchius*-like haplotypes in *N. chlorobranchius*, indicating a more recent divergence from those haplotypes in *N. rufilneatus*. In addition, hybrid museum specimens with *N. camurus* and *N. rufilineatus* as the parental species have been sampled from the rivers, and some of the exact localities, where these haplotypes were sampled (Fig. 4.14) (Chapter 2) and *N. chlorobranchius* does not occur in these rivers. The *chlorobranchius*-like haplotype observed in *N. camurus* that is most similar to those observed in *N. chlorobranchius* is from the Nolichucky River system, where introgression has been documented with allozymes (Eisenhour 1995) and museum hybrid specimens of *N. camurus* X *N. chlorobranchius* have been sampled (Chapter 2). The closer similarity of the *chlorobranchius*-like haplotypes observed in *N. camurus* to the *chlorobranchius*-like haplotypes observed in *N. rufilineatus*, geographic distribution of the *chlorobranchius*-like haplotypes observed in *N. camurus*, and the *N. camurus* X *N. rufilineatus* hybrid museum specimens support a hypothesis of *N. rufilineatus* acting as a conduit species for *chlorobranchius*-like haplotypes into *N. camurus*.

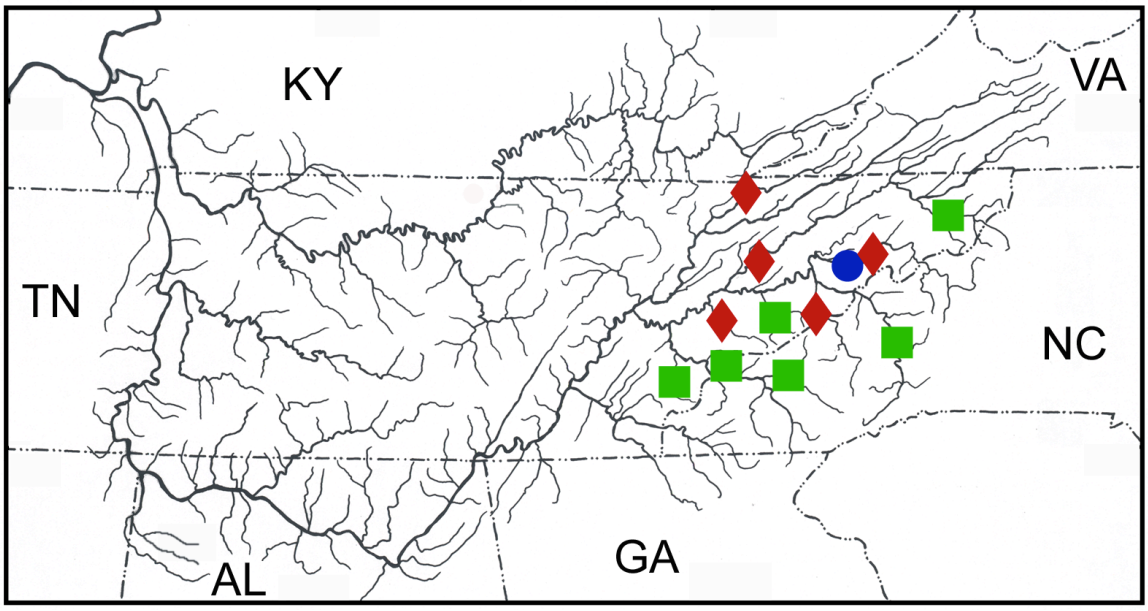

Fig. 4.14. Map of the Cumberland and Tennessee Rivers with collection localities marked where hybrid specimens resulting from a cross of two of the three focal species (Chapter 2). Red diamonds indicate *N. camurus* X *N. rufilineatus*, green squares indicate *N. chlorobranchius* X *N. rufilineatus*, and the blue circle indicates *N. camurus* X *N. chlorobranchius*.

Patterns of Introgression among Darters

We identified three patterns in the process of describing the introgression in *Nothonotus* darters: 1) asymmetric introgression of mitochondrial genomes with little evidence of nuclear introgression, 2) mitochondrial donors have larger and smaller range sizes than recipient species, and 3) more than one species can be the donor when multiple species are involved in mitochondrial replacement. The first result is not unexpected given the pattern's frequency in both darters and more inclusive clades (Bossu and Near accepted; Chan and Levin 2005; Piller et al. 2008; Ray et al. 2008) and we described this pattern in the discussion of *Nothonotus* above. Asymmetric introgression has many potential causes, mentioned in the introduction, but narrowing the possibilities requires experimentation. The second and third patterns are not consistent with those observed in other darter clades (Bossu and Near accepted; Piller et al. 2008; Ray et al. 2008; Williams et al. 2007). However, these differences may be influenced by factors not encountered or confounded in the previous studies. Additionally, the contributing factors can be experimentally tested and the isolating barriers that may be breaking down can be identified.

Range size discrepancy is fairly consistent across darter clades, but one case in *Nothonotus* is counter to the trend and this may be explained by a characteristic exclusive to that case. In other darter subclades the donor species has a larger range than the recipient species (Bossu and Near accepted; Piller et al. 2008; Ray et al. 2008; Williams et al. 2007), but this is not consistently repeated in *Nothonotus*. *Nothonotus camurus* has a larger range than *N. rufilineatus*, but *N. chlorobranchius* has a smaller range than *N. rufilineatus*. Most donating species have larger range sizes and this indicates that a characteristic promoting large range sizes is itself, or is correlated with, a characteristic that promotes asymmetric introgression. For instance, species with less selective females may be able to maintain larger ranges and this could lead to asymmetric sexual isolation (Mendelson 2003a; Wirtz 1999). Introgression in *N. rufilineatus* and *N. chlorobranchius* may run counter to this trend because *N. chlorobranchius* is a much larger species than *N. rufilineatus* (Etnier and Starnes 1993) and in a hybrid viability study in another clade of perciform fish the larger species was the better maternal parent (Bolnick and Near 2005). In the other studies, and in *N. camurus* and *N. rufilineatus*, the size difference is relatively small or the larger species also had the larger range. The inconsistency in *Nothonotus* indicates that certain characteristics, e.g. body size, may have more influence on the outcomes of introgression than others, and that experiments comparing hybrid viabilities and mate choice trials could test the relative contributions of each.

The third pattern of one species being the universal donor when multiple species are involved in mitochondrial replacement is not consistent in *Nothonotus*, but this discrepancy may be caused by problems in taxon recognition and, if this is accounted for, the patterns are consistent. Bossu and Near (accepted) document multiple instances of introgression of *Etheostoma caeruleum* mitochondrial genomes into various species in the *Etheostoma spectabile* species complex and, if *E. caeruleum* is a single taxon, there is a pattern of one donor species. However, there are two Ph.D. dissertations that describe distinct lineages and species within *E. caeruleum* (Knapp 1964; McCormick 1990), but the unpublished status precludes formal recognition of these taxa. Accounting for these additional taxa in Bossu and Near (accepted) makes the pattern consistent with the

pattern in *Nothonotus*. Instead of one species being the universal donor, species in the same clade are exclusively donors or recipients when multiple clades are involved in mitochondrial replacement. In *Nothonotus*, the two mitochondrial donors, *N. camurus* and *N. chlorobranchius*, are in a clade with *N. bellus* and this clade is fairly divergent from the mitochondrial recipient, *N. rufilineatus* (Keck and Near 2008). The pattern of clades being exclusive donors or recipients indicates that a characteristic common to each of the taxa in the donor or the recipient clade promotes asymmetric introgression, as well as a phylogenetic effect on likelihoods of introgression.

Mitochondrial replacement in *Nothonotus* is fairly consistent with patterns observed across darter clades and in other animal clades, and these patterns provide insights into the failure of reproductive isolating barriers in vertebrates. We identified extensive introgression in *Nothonotus* involving multiple species both in the past and contemporaneously. Patterns of introgression in *Nothonotus* and other darter clades is generally restricted to directional mitochondrial exchange with limited nuclear exchange, the donating species typically has a larger range than the recipient species, and in cases of mitochondrial introgression involving multiple species the donors and recipients are in exclusive clades. Common characteristics of the taxa involved in introgression indicate experimental studies of hybrid viability and mate choice trials could help identify the major contributing mechanisms to the breakdown of reproductive isolating barriers in darters.

CHAPTER 5

A young clade repeating an old pattern: Diversity in *Nothonotus* darters (Teleostei: Percidae) endemic to the Cumberland River[1]

ABSTRACT

Phylogeographic hypotheses of diversification in eastern North American freshwater fishes have primarily relied on divergence between major centers of diversity and major river systems. However, these hypotheses do not fully explain the rich diversity within these regions and river systems and this is true for darters (Percidae: Etheostomatinae), a diverse and colorful clade of teleost fishes endemic to eastern North America. Relatively old divergences on small geographic scales have been observed in one subclade of darters, barcheeks, that occur in the Cumberland River in Kentucky and Tennessee, but it is unknown if this pattern is consistent in other darter subclades. To make a comparison we explored the diversity in two *Nothonotus* darters, *N. microlepidus* and *N. sanguifluus*, endemic to the Cumberland River, using three methods of delimiting lineages (gene trees, estimated species trees, and morphometrics). The results of the gene tree and morphometric analyses indicate that the current taxonomy does not describe the actual diversity. Four distinct lineages were evident, despite retained ancestral polymorphism obscuring genetic signals of divergence and lack of samples from extirpated populations. The pattern of divergence on a relatively small geographic scale in the Cumberland River system is consistent across old and young darter subclades, barcheeks, and *Nothonotus*, respectively, indicating the persistence of isolating mechanisms in the Cumberland River through significant evolutionary time. Additionally, the highly structured nature of Cumberland River darter populations, and the potential for this pattern to occur in more disparate clades or in other geologically stable areas, should be considered in conservation and comparative studies.

INTRODUCTION

Phylogeographic hypotheses of diversification in the spectacular eastern North American freshwater fish fauna have been primarily based on the relationships between allopatrically distributed clades occurring in major centers of diversity and river systems (Hocutt and Wiley 1986; Mayden 1988; Near and Keck 2005; Near et al. 2001; Ray et al. 2006; Wiley and Mayden 1985). However, diversity in eastern North American fish clades, particularly darters (Percidae: Etheostomatinae), is not always fully explained by divergence between these regions and there are often multiple distinct lineages within major river systems (Ceas and Page 1997; Hardman 2004; Hollingsworth and Near in press; Keck and Near 2008; Page et al. 1992; Page et al. 2003; Page and Near 2007). Only recently has a study on freshwater teleost fish explicitly tried to identify the temporal and geographic scale of diversification within one of these major drainages, the Cumberland River (Hollingsworth and Near in press).

[1] A modified version of this chapter to be submitted as: Keck, B.P. and T.J. Near. A young clade repeating an old pattern: Diversity in *Nothonotus* darters (Teleostei: Percidae) endemic to the Cumberland River. *Molecular Ecology*. My contributions to this manuscript include: 1) development of ideas, 2) data collection and analyses, 3) most of the writing.

More endemic darter species occur in the Cumberland River per drainage area than in either the Tennessee River or the Mobile Basin, despite having the lowest total number of endemic darters: one endemic darter per 6175 km^2 in the Tennessee River, one per 5500 km^2 in the Mobile Basin, and one per 3375 km^2 in the Cumberland River. Accordingly, a recent study on barcheek darters by Hollingsworth and Near (in press)identified divergence within the Cumberland River at relatively small geographic scales. Relationships between recognized barcheek species were well resolved, with a crown node age estimate of 16.6 million years, and several species had striking amounts of intraspecific divergence among lineages from different river systems (Hollingsworth and Near in press). In fact, the divergence time estimates between five populations within the Caney Fork River (Fig. 5.1), a river system only 4587 km^2, range between 2 and 8 million years old. Hollingsworth and Near (in press) conclude that the mechanisms responsible for divergence at such small scales are also responsible for the maintenance of that isolation over millions of years to the present. If the observed small geographic scales of divergence are driving the high rates of endemism in the Cumberland River, this pattern should be repeated in other clades.

In our recent work on *Nothonotus* darters (Percidae: Etheostomatinae) we found a close relationship between two Cumberland River endemics, *N. microlepidus* and *N. sanguifluus*, (Keck and Near 2008; Near and Keck 2005) that had not been identified in previous studies incorporating these two species (Etnier and Williams 1989; Raney and Zorach 1967; Wood 1996; Zorach and Raney 1967). In addition, the *N. sanguifluus* from the Caney Fork River (Fig. 5.1) formed a clade that was sister to all other *N. sanguifluus* and *N. microlepidus* plus *N. maculatus* in the mitochondrial gene tree (Keck and Near 2008), rendering these species, as currently recognized, non-monophyletic in mitochondrial or nuclear gene trees (Keck and Near 2008; Near and Keck 2005). Trees derived from the mitochondrial gene cyt*b* had a clade of *N. microlepidus* and *N. sanguifluus* sharing a Most Recent Common Ancestor (MRCA) with *N. maculatus* and this clade shared a MRCA with the Caney Fork River *N. sanguifluus* (Keck and Near 2008; Near and Keck 2005). In the nuclear encoded S7 intron tree all *N. microlepidus* and *N. sanguifluus* formed a clade with a significant Bayesian posterior probability (> 0.94), but within clade resolution was poor (Keck and Near 2008). The identification of the *N. microlepidus – N. sanguifluus* relationship indicates that there is at least one *Nothonotus* divergence within the Cumberland River system, but identification of distinct lineages is obscured by discordant gene trees (Keck and Near 2008; Near and Keck 2005).

Species paraphyly and polyphyly are often the result of cryptic species, retained ancestral polymorphism, or introgression (Funk and Omland 2003), and the former two are more likely in *Nothonotus*. Hybridization has been documented in *Nothonotus* in Chapters 2 and 4 and by Eisenhour (1995), but does not seem likely in this instance as these two gaudily colored darters have completely allopatric distributions in the Cumberland River (Fig. 5.1). Moreover, *N. microlepidus* and *N. sanguifluus* are in the subclade of *Nothonotus* distinguished by their male egg guarding behavior, the *N. maculatus* clade (Near and Keck 2005), and there are no known hybrid specimens resulting from natural hybridization of two members of the *N. maculatus* clade (Chapter 2). The crown node age estimate for the *N. microlepidus* and *N. sanguifluus* clade is only 1.4 million years and this relatively young age supports a hypothesis of retained ancestral

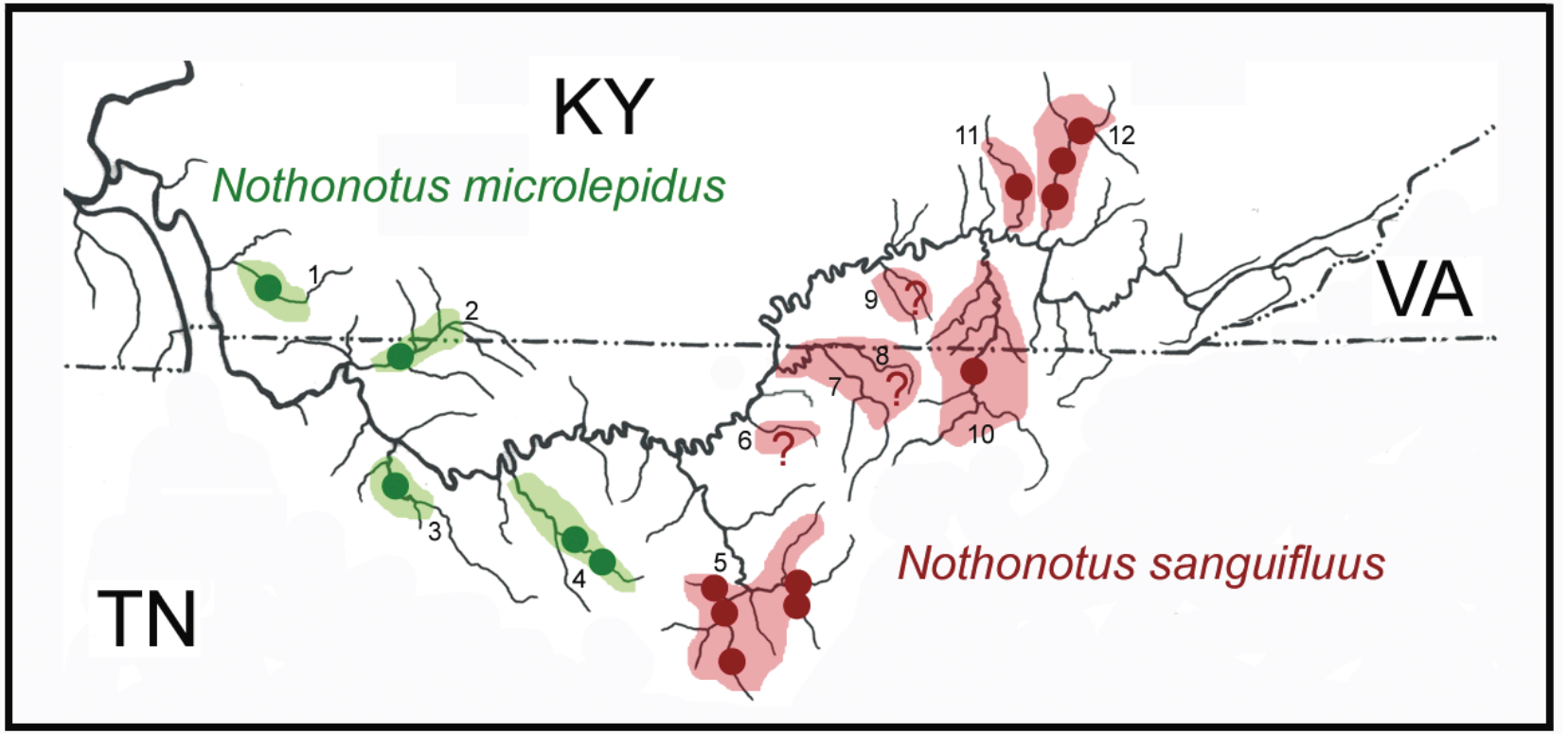

Fig. 5.1. Map of the Cumberland River and tributaries with distributions (shading) and sampling localities (dots) of *N. microlepidus* (green) and *N. sanguifluus* (red). Tributaries are: **1** – Little River, **2** – Red River, **3** – Harpeth River, **4** – Stones River, **5** – Caney Fork River, **6** – Roaring River, **7** – Obey River, **8** – Wolf River, **9** – Otter Creek, **10** – Big South Fork, **11** – Buck Creek, **12** – Rockcastle River.

polymorphism because there has not been sufficient time relative to populations sizes for sorting to occur despite genetic isolation (Avise 2004; Carstens and Knowles 2007; Hudson and Coyne 2002; Hudson and Turelli 2003; W.P. Maddison 1997; W.P. Maddison and Knowles 2006; Pamilo and Nei 1988; Rosenberg 2002; 2003; Takahata 1989). Small geographic scales of darter divergence in the Cumberland River (Hollingsworth and Near in press) and a relatively young clade make unrecognized diversity, ancestral polymorphism, or a combination of the two better candidates for explaining the observed paraphyly.

Discordant genealogies are not devoid of phylogenetic information, and recently developed methods that incorporate coalescent theory have been shown to extract this information (Belfiore et al. 2008; Carstens and Knowles 2007; Degnan and Salter 2005; S. V. Edwards et al. 2007; Knowles and Carstens 2007; Liu and Pearl 2007; Liu et al. 2008; W.P. Maddison and Knowles 2006). However, there are many methods of delimiting species and each method may yield different results for the same taxa (Sites and Marshall 2004). Therefore, we used three methods to identify *Nothonotus* diversity in the Cumberland River, including Bayesian analysis of individual gene and concatenated datasets, a Bayesian method of estimating species trees (BEST) (S. V. Edwards et al. 2007; Liu et al. 2008), and analyses on 10 meristic characters. We address two primary questions in this study: 1) How many lineages can be identified? and 2) Is the pattern consistent with diversification on a small geographic scale? After answering these two questions we discuss the implications for Cumberland River fauna and darters in general.

METHODS

Study System

Nothonotus is a subclade of darters containing 20 recognized species distributed throughout eastern North America and has a crown node age estimate of approximately 19 million years (Near and Keck 2005). These sexually dichromatic species prefer the faster flowing portions of streams and are often sympatric with other darters, including other *Nothonotus* (Etnier and Starnes 1993; Kuehne and Barbour 1983; Page 1983). Hypotheses of *Nothonotus* relationships have been based on: 1) morphology (Etnier and Williams 1989); 2) morphology, behavior, and allozymes (Wood 1996); 3) mitochondrial DNA sequence data (Near and Keck 2005); and 4) mitochondrial and nuclear DNA sequence data and morphology (Keck and Near 2008). Analyses based on morphology and allozymes either did not find the *N. microlepidus – N. sanguifluus* sister species relationship (Wood 1996) or they were included in unresolved clades containing the other members of the *N. maculatus* clade (Etnier and Williams 1989).

The distribution of *N. microlepidus* includes several tributaries of the Cumberland River and these are the Little River, Red River, Harpeth River, and Stones River (Fig. 5.1). *Nothonotus microlepidus* is presumed extirpated from the type locality on the Stones River, due to the construction of a dam (D.A. Etnier, pers. comm.). The historical distribution of *N. sanguifluus* includes the Caney Fork River above the falls, Roaring River, Obey River, Wolf River, Otter Creek, Buck Creek, Rockcastle River, and Big South Fork Cumberland River (Fig. 5.1). Cope (1870) gave only a general suggestion as to the type locality with "This lovely species is common in the head waters of the South Fork Cumberland, in Tennessee."

The meristics of *N. microlepidus* and *N. sanguifluus* have never been directly compared as sister taxa and variation within the latter has never been explored. The color differences between *N. microlepidus* and *N. sanguifluus* have been compared in the description of *N. microlepidus* by Raney and Zorach (1967) and also in a systematic study of *N. maculatus* (Zorach and Raney 1967). The most notable color differences were that *N. microlepidus* has deep green in its median fins (dorsal, caudal, and anal) and pelvic fins, where *N. sanguifluus* has a blood red color in the median fins. However, Raney and Zorach (1967) considered *N. rubrus* to be the sister species of *N. microlepidus* and made all meristic comparisons to it. Cope's (1870) description of *N. sanguifluus* (as *Poecilichthys sanguifluus*) makes no comparisons to other species, but does mention possible affinities to *N. camurus*, *N. vulneratus*, and *N. maculatus*. In Zorach and Raney's (1967) diagnosis of *N. sanguifluus* as a subspecies of *N. maculatus*, *N. sanguifluus* is lumped into a single population for meristic comparisons to *N. maculatus* and *N. vulneratus* populations partitioned by river systems.

Collection and Analysis of Sequence Data

We collected fishes using seines or backpack electrofishing units. We preserved tissue biopsies in 100% ethanol and deposited them into the Yale Fish Tissue Collection (YFTC). We used corresponding labels stored with the tissue biopsies and attached to voucher specimens for reference. We preserved voucher specimens by fixation in 10% formalin for at least seven days, a tap water wash for two days, and stored in 70% ethanol. Respective museum staff deposited the voucher specimens into the University of Tennessee Research Collection of Fishes (UT) or the Yale Peabody Museum of Natural History Fish Collection (YPM). We sampled *N. microlepidus* and *N. sanguifluus* from throughout their known ranges (Fig. 5.1). Our focal species are in the *N. maculatus* clade (Keck and Near 2008) within *Nothonotus*, a subclade of darters (Percidae: Etheostomatinae), and all members of this clade were sampled with multiple individuals (Table D.1). Outgroup taxa included six other *Nothonotus*, three non-*Nothonotus* darters, and one non-darter percid (Table D.1).

We isolated nucleic acids using Qiagen DNAeasy kits according to the manufacturer's protocol. The mtDNA encoded cytochrome *b* (cyt*b*), nuclear encoded first intron of the S7 ribosomal protein, nuclear encoded intron of RAG1, and nuclear encoded MLL genes were amplified using primers and PCR protocols outlined in; cyt*b* (Near et al. 2000), S7 (Chow and Hazama 1998), RAG1 and MLL (Bossu and Near *accepted*). We cleaned successful PCR products for sequencing using Qiagen Qiaquick PCR purification kits according to the manufacturer's protocol, or by digestion with 1.0 unit Exonuclease I and Shrimp Alkaline Phosphate and incubated for 15 min at 37 ^{o}C and 20 min at 80 ^{o}C, or by Polyethylene Glycol (PEG) precipitation. In PEG an equal volume (usually ~23 µl) of 20% PEG solution was added to PCR products, vortexed briefly, and placed in a 37 ^{o}C incubation chamber for 15 min. After incubation, the solution was placed in a centrifuge at ~6000 rcf for 20 min, the supernatant removed, and the remaining DNA was washed with 200 µl of 70% ethanol. The ethanol was evaporated from the DNA using a vacuum centrifuge for ~15 min and the DNA was re-suspended in ~18 µl of H_2O. Sequencing was done by the DNA Sequencing Facility on Science Hill at Yale University or WM Keck Foundation Biotechnology Resource Laboratory at Yale University. We assembled

contiguous sequences from individual sequence reaction files in Sequencher (Gene Codes, Ann Arbor, MI, USA). We aligned sequences for cyt*b* and RAG1 by eye as there were no differences in sequence length between individuals and used ClustalX (Thompson et al. 1997) to align S7 and MLL sequences. We deposited all sequences not used in previous studies (Keck and Near 2008; Near and Keck 2005) or Chapter 4 in GenBank with the accession numbers XXXXXX-XXXXXX (to be released when published). We removed identical haplotypes and alleles using MacClade (D.R. Maddison and Maddison 2000) for certain analyses, and only if all three or four genes had the same haplotype for the concatenated nuclear and nuclear/mitochondrial datasets.

We used the computer program MrBayes 3.1 to create phylogenies using partitioned mixed-model Bayesian analyses (Ronquist and Huelsenbeck 2003) with posterior probabilities estimated using metropolis-coupled Markov chain Monte Carlo (Huelsenbeck et al. 2001; Larget and Simon 1999). We partitioned datasets by codon position for coding sequences and as a single partition for non-coding sequences. We determined the optimal maximum likelihood model of molecular evolution for each partition using AIC as executed in the computer program Modeltest 3.0 (Posada and Crandall 1998). We ran six separate analyses; one for each gene individually, one on a concatenated dataset including the three nuclear genes, and one on a concatenated dataset including the three nuclear genes and the mitochondrial gene. We ran each analysis for 10 million generations on the computer cluster at the Computational Biology Service Unit at Cornell University. We discarded the first 2 million generations as burn-in and the posterior probabilities of nodes were calculated from the remaining post burn-in trees.

Concatenation methods can result in relationships that are over supported and even misleading when the evolutionary histories of genes are disparate, there is unsorted ancestral polymorphism, or there is low taxon sampling (Degnan and Rosenberg 2006; Kubatko and Degnan 2007), and the first two are more likely in recently diverged lineages. However, probabilistic models that incorporate coalescent theory (Liu and Pearl 2007) have been shown to better estimate species trees under these conditions (Belfiore et al. 2008; S. V. Edwards et al. 2007; Liu et al. 2008). We used the computer program BEST (Liu and Pearl 2007), a modified version of MrBayes, to estimate species trees from the multi-locus dataset with multiple alleles per taxon as in Belfiore et al. (2008). We partitioned the datasets for BEST analysis by locus and not by codon position as in the MrBayes runs above. BEST assumes no reticulation or retained ancestral polymorphism, so relationships between taxa with these conditions will have lower support than between taxa with monophyletic alleles. We created six datasets with *N. microlepidus* and *N. sanguifluus* divided into varying numbers of taxa to assess resolution at different levels of taxon partitioning. We used a single individual of six other *Nothonotus* in addition to no more than two individuals of *N. microlepidus* and *N. sanguifluus* from each of the river drainages. We based the taxa partitions on currently recognized species, gene trees in this study, and geographic patterns of diversity in barcheek darters (Hollingsworth and Near in press). The partitions of *N. microlepidus* and *N. sanguifluus* were: 1) current taxonomy – *N. microlepidus* and *N. sanguifluus* as two taxa, 2) Caney Fork – same as previous, but with Caney Fork *N. sanguifluus* as a separate taxon, 3) Caney Fork and Big South Fork – same as previous, but with Big South Fork *N. sanguifluus* as a separate taxon, 4) *N. sanguifluus* by river – *N. sanguifluus* from each

river as a separate taxon and *N. microlepidus* as a single taxon, and 5) All River – *N. microlepidus* and *N. sanguifluus* from each river as separate taxa. We set each BEST analysis to run for at least 150 million generations. We used Tracer 1.4 (Rambaut and Drummond 2003) to visualize parameter convergence during and after the BEST runs.

Allele networks were created using median joining methods (Bandelt et al. 1999) in the software Network 4.5 (fluxus-engineering.com). Invariant sites were removed from the datasets in PAUP 4.0 (Swofford 2003) before running the analyses in Network 4.5. The datasets used in the network analyses contained identical haplotypes and alleles. A network was created for each of the four genes: the cyt*b* network contained all individuals of *N. maculatus*, *N. microlepidus*, and *N. sanguifluus*, and the S7, MLL, and RAG1 networks contained all individuals of the *N. maculatus* species group, plus *N. moorei* and *N. rubrus*.

Collection and Analysis of Meristic Data

Morphological characters, particularly scale, fin ray, and fin spine counts (meristics), have frequently been used by ichthyologists to differentiate and delimit closely related taxa (Etnier and Starnes 1993; C.L. Hubbs and Lagler 1958). We collected and preserved *N. microlepidus* and *N. sanguifluus* from throughout their ranges for meristic analysis as described above for the collection of genetic data (Table D.2). We augmented these samples with data collected from specimens in UT, YPM, and the Cornell University Museum of Vertebrates (CUMV)(Table D.2). We collected meristic data for characters commonly used to differentiate fish species as outlined in Hubbs and Lagler (1958) and Page (1974b; 1981) including: 1) pored lateral line scales, 2) transverse scales from anterior base of first dorsal fin to lateral line, 3) transverse scales from lateral line to anterior base of anal fin, 4) scales at minimum depth of the caudal peduncle, 5) scales on the opercle, 6) spines in the first dorsal fin, 7) rays in the second dorsal fin, 8) rays in the anal fin, 9) rays in the pectoral fin, and 10) rays in the pelvic fin. We used all specimens in each museum lot unless the lot was particularly large (>70 individuals) or if an individual was damaged or less than 2.5 cm.

We used Discriminant Function Analysis (DA) in the statistical program SPSS 16 (© 2007 SPSS Inc.) to test whether individuals could be classified into groups as predicted using the meristic characters. We also used SPSS 16 to check for normality (K-S test), outliers (box plots and z-scores), and homogenous variance matrices (Box's M). Some of the populations of *N. sanguifluus* were not partitioned as distinct taxa, because they had fewer individuals than variables and these are the only known specimens. Prior probabilities in each run were set proportionally. We assigned groups based on current taxonomy and the results of the genetic analyses above: 1) current taxonomy – *N. microlepidus* and *N. sanguifluus* as two taxa, 2) Caney Fork – same as previous, but with Caney Fork *N. sanguifluus* as a separate taxon, 3) Caney Fork and Big South Fork – same as previous, but with Big South Fork *N. sanguifluus* as a separate taxon, and 4) genetic – three taxa partitioned by Caney Fork and Big South Fork *N. sanguifluus* as separate taxa and all other *N. sanguifluus* and all *N. microlepidus* as a single taxon.

RESULTS

Genetic Analyses

We obtained sequence data from all populations of *N. microlepidus*, but were unable to obtain specimens from four populations of *N. sanguifluus* (Fig. 5.1 and Table D.1). We made multiple attempts to find individuals from these rivers at several localities over four years, but were unsuccessful. Aligned sequence length for the four genes ranged from 550 base pairs for S7 to 1319 base pairs for RAG1 and both focus species had multiple alleles for each gene (Table 5.1). We obtained optimal models of molecular evolution for each data partition using Modeltest and used these for analyses in MrBayes and BEST (Table D.3). Genetic diversity for each gene within *N. microlepidus*, *N. sanguifluus* as one group, *N. sanguifluus* from the Caney Fork, *N. sanguifluus* from the Big South Fork, *N. sanguifluus* from the Rockcastle, and *N. sanguifluus* from Buck Creek, is given in Table 5.2. The genetic diversity in cyt*b* is much higher for *N. sanguifluus* as one population than that of *N. microlepidus*, and when *N. sanguifluus* populations are considered, diversity in cyt*b* is more similar to that in *N. microlepidus* (Table 5.2). All *N. sanguifluus* have much lower MLL diversity than *N. microlepidus*, and the Caney Fork, Big South Fork, and Rockcastle *N. sanguifluus* had no diversity in MLL alleles (Table 5.2). The Caney Fork *N. sanguifluus* had more genetic diversity in S7 and RAG1 alleles than the other populations of *N. sanguifluus* (Table 5.2).

Nothonotus was monophyletic in every analysis run in MrBayes and relationships with non-*Nothonotus* included in the analyses are not shown in the figures (Figs. 5.2, 5.3, and 5.4). Bayesian analysis of the cyt*b* sequences resulted in a fairly well resolved tree, similar to cyt*b* trees in previous studies of *Nothonotus* relationships (Fig. 5.2)(Keck and Near 2008; Near and Keck 2005). The *N. maculatus* clade was monophyletic and supported by a significant Bayesian posterior probability. A significantly supported clade including a monophyletic *N. maculatus* within a clade of non-monophyletic *N. microlepidus* and *N. sanguifluus* was recovered. We also found Caney Fork River *N. sanguifluus* as a clade (non-significant support) with a basal relationship to the *N. maculatus* lineage and all other *N. sanguifluus* and *N. microlepidus*. Three of the four Big South Fork *N. sanguifluus* haplotypes formed a significantly supported clade basal to the other Big South Fork *N. sanguifluus* haplotype and all haplotypes from Buck Creek and Rockcastle River *N. sanguifluus* and all *N. microlepidus*. One haplotype was shared between Big South Fork and Buck Creek *N. sanguifluus* and all other Buck Creek haplotypes form a significantly supported clade with this haplotype. Another haplotype is shared between Rockcastle River *N. sanguifluus* and Red River *N. microlepidus*; this haplotype was isolated from three *N. sanguifluus* individuals and one *N. microlepidus* individual.

The three nuclear gene trees and concatenated nuclear tree were much less resolved than the mitochondrial cyt*b* tree (Fig. 5.3). None of the trees resolved a monophyletic egg guarding *N. maculatus* species group, although the RAG1, MLL, and concatenated trees had a significantly supported *N. maculatus* species group inclusive of *N. moorei* and *N. rubrus*. Of the species with multiple unique S7 alleles, only *N. maculatus* was monophyletic in the S7 tree. Relationships between *N. microlepidus* and *N. sanguifluus* were largely unresolved and none was significantly supported. One S7 allele was shared between *N. microlepidus* and *N. sanguifluus*; this allele was isolated

Table 5.1. Number of individuals sequenced, unique haplotypes and alleles observed and aligned sequence length per gene for *N. microlepidus*/*N. sanguifluus*.

Gene	# Individuals	# Haplotypes or Alleles	Sequence Length
cyt*b*	19/35	9/24	1140 bp
MLL	19/30	6/3	720 bp
S7	16/30	15/19	550 bp
RAG1	16/30	10/12	1319 bp

Table 5.2. Genetic distances given as percentages for each gene in *N. microlepidus* and *N. sanguifluus*. Average genetic distance is the top number and maximum genetic distance is the lower number (minimum genetic distance was 0 for all groups).

	Gene			
Species	cyt*b*	S7	MLL	RAG1
N. microlepidus	0.34	0.53	0.03	0.07
	0.61	1.54	0.28	0.23
N. sanguifluus	1.38	0.49	0.01	0.09
All	2.54	1.53	0.14	0.30
N. sanguifluus	0.33	0.32	0.00	0.09
Caney Fork	0.70	0.77	0.00	0.23
N. sanguifluus	0.07	0.11	0.00	0.03
Big South Fork	0.26	0.38	0.00	0.07
N. sanguifluus	0.27	0.06	0.00	0.05
Rockcastle	0.88	0.38	0.00	0.15
N. sanguifluus	0.23	0.15	0.05	0.04
Buck Creek	0.44	0.38	0.14	0.07

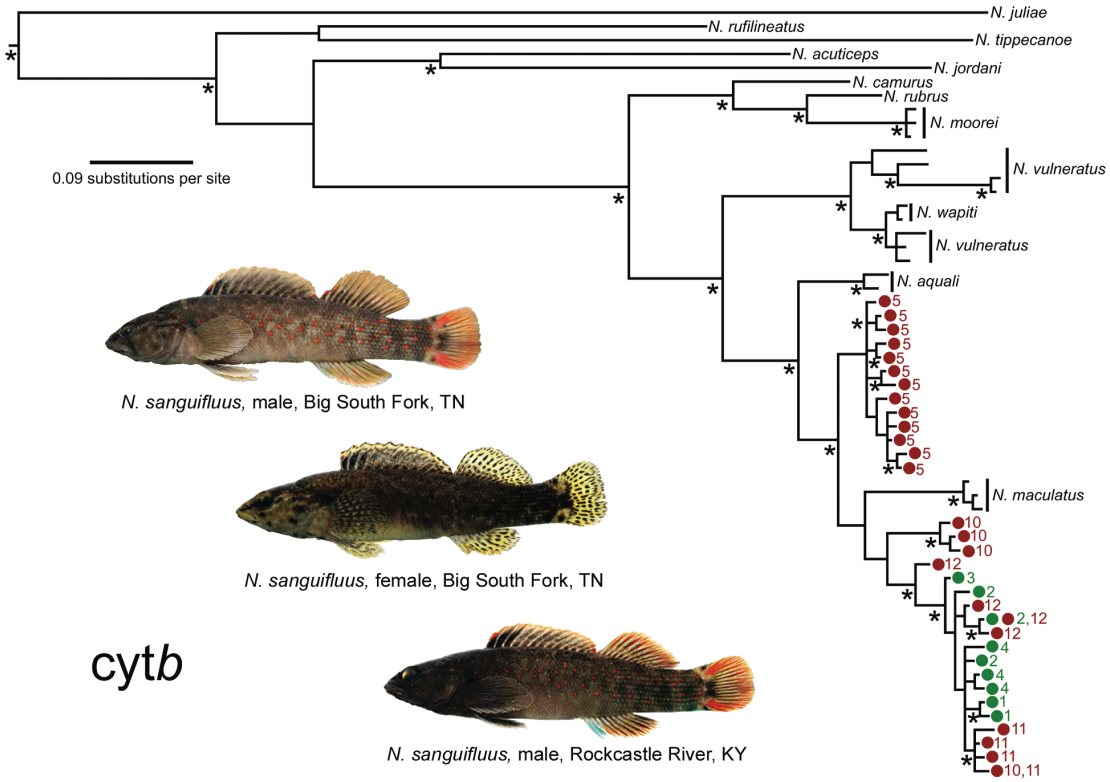

Fig. 5.2. Phylogeny resulting from Bayesian analysis of the mitochondrial cytochrome *b* dataset. All nodes supported with a Bayesian posterior probability of > 0.94 are indicated with an asterisks. Green dots indicate haplotypes observed in *N. microlepidus*, red dots indicate haplotypes observed in *N. sanguifluus*, tips with dots of both colors indicate the haplotype was observed in both species. The numbers following the dots correspond to the numbers for each Cumberland River tributary in Fig. 5.1 and indicate the tributaries the individuals containing that haplotype were sampled from.

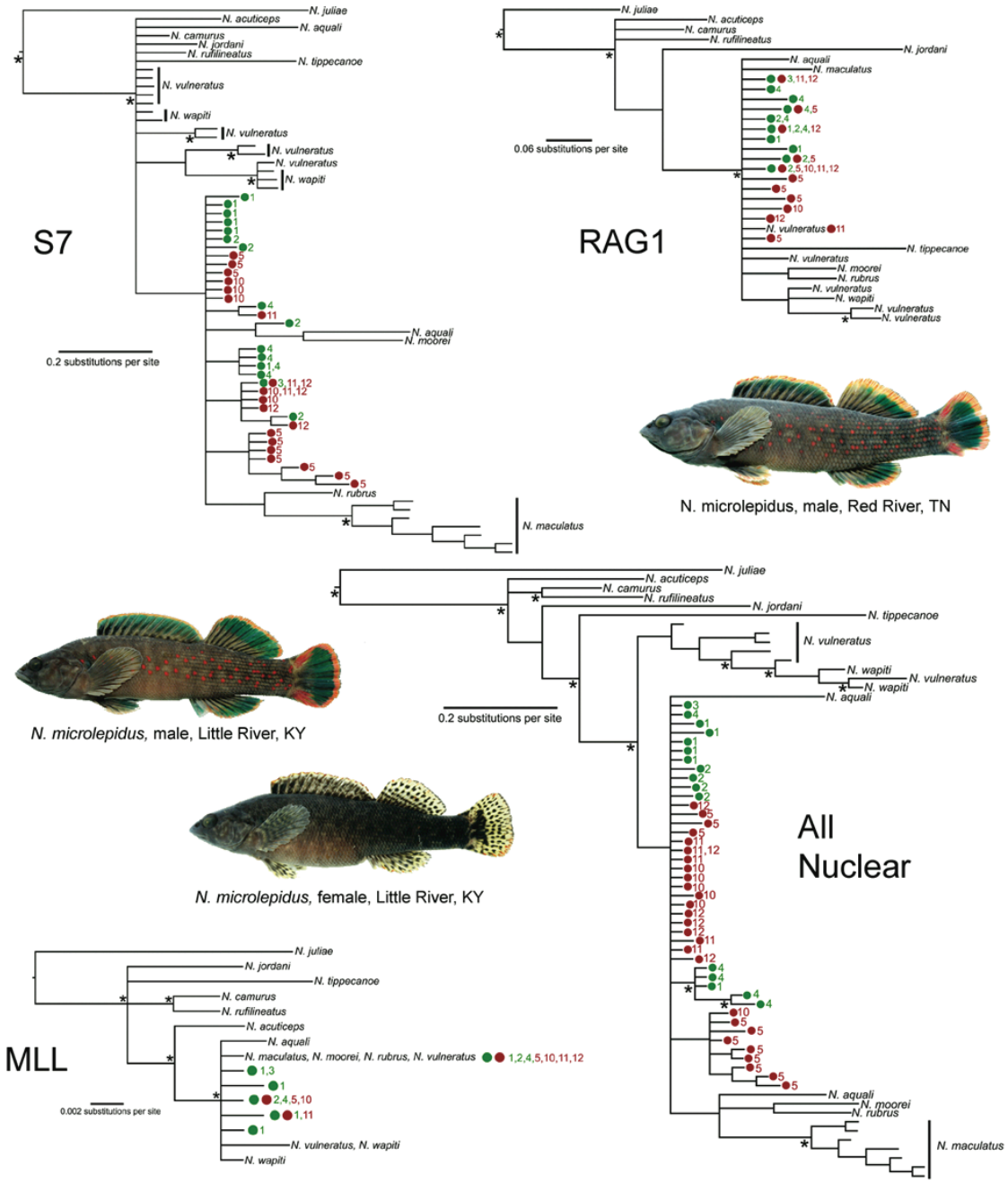

Fig. 5.3. Phylogeny resulting from Bayesian analysis of the three nuclear genes (S7, RAG1, MLL) independently and concatenated. All nodes supported with a Bayesian posterior probability of > 0.94 are indicated with an asterisks. Green dots indicate alleles observed in *N. microlepidus*, red dots indicate alleles observed in *N. sanguifluus*, tips with dots of both colors indicate the allele was observed in both species. The numbers following the dots correspond to the numbers for each Cumberland River tributary in Fig. 5.1 and indicate the tributaries the individuals containing that allele were sampled from.

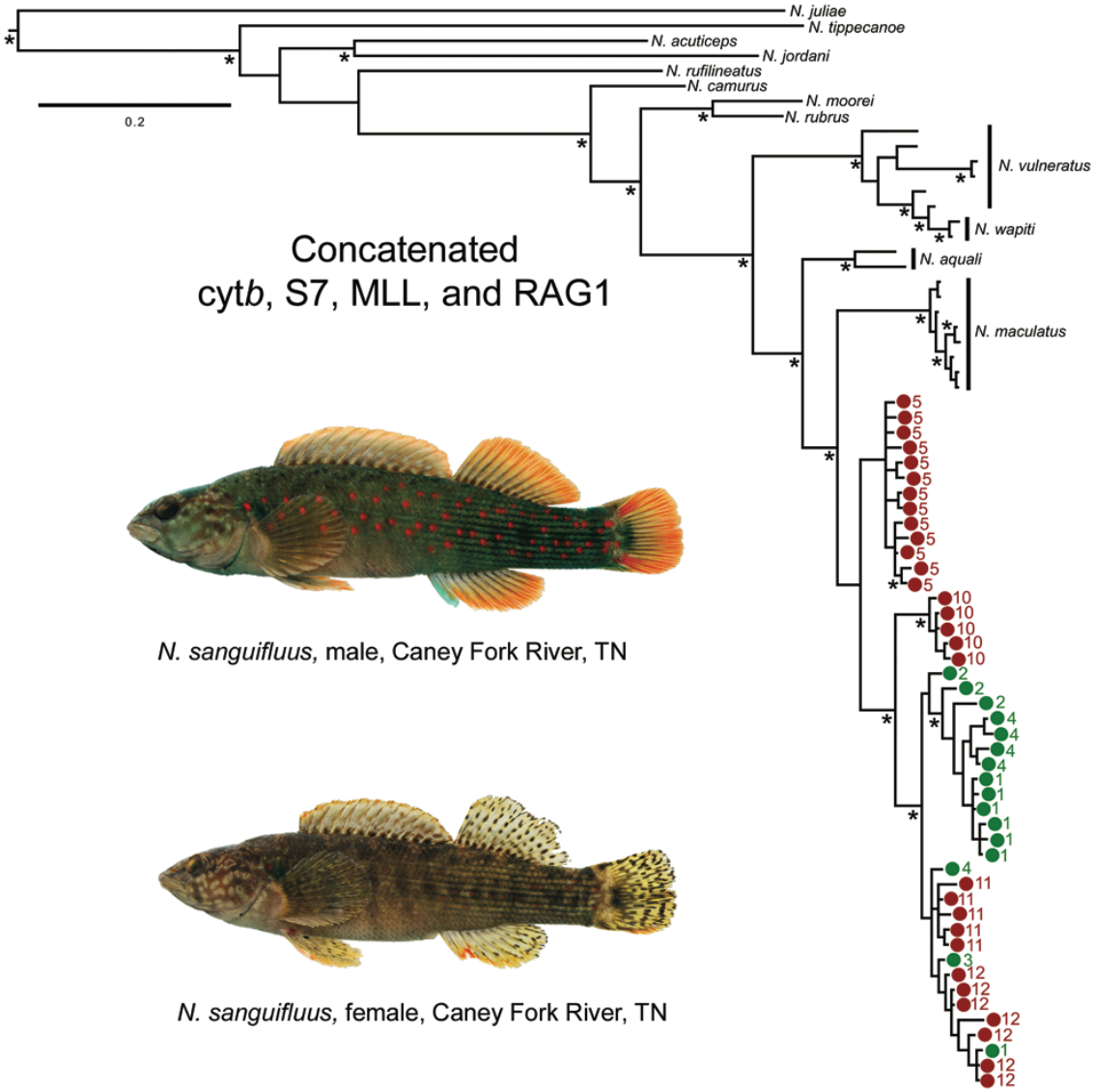

Fig. 5.4. Phylogeny resulting from Bayesian analysis of the concatenated dataset including cyt*b* and the three nuclear genes. All nodes supported with a Bayesian posterior probability of > 0.94 are indicated with an asterisks. Green dots indicate haplotypes observed in *N. microlepidus*, red dots indicate haplotypes observed in *N. sanguifluus*, tips with dots of both colors indicate the haplotype was observed in both species. The numbers following the dots correspond to the numbers for each Cumberland River tributary in Fig. 5.1 and indicate the tributaries the individuals containing that haplotype were sampled from.

from one Harpeth River *N. microlepidus*, one Rockcastle River *N. sanguifluus*, and three Buck Creek *N. sanguifluus*. Seven of the 10 unique S7 alleles from Caney Fork River *N. sanguifluus* formed a clade without significant support. We found the fewest unique alleles from MLL and there was no resolution between any members of the *N. maculatus* species group. In fact, one MLL allele was found in all *N. maculatus*, all *N. moorei*, all *N. rubrus*, five of six *N. vulneratus*, *N. microlepidus* individuals from the Little River, Red River, and Stones River, and *N. sanguifluus* individuals from all populations. *Nothonotus microlepidus* had three alleles that were not found in any other species and three alleles that were only shared with *N. sanguifluus*. None of the species with multiple unique RAG1 alleles were monophyletic in the RAG1 tree and there was very little resolution between the members of the *N. maculatus* species group. No populations of *N. microlepidus* or *N. sanguifluus* contained monophyletic alleles and they were often found in multiple populations. There were more significantly supported relationships in the concatenated nuclear dataset than any nuclear gene analyzed independently. *Nothonotus maculatus* was the only species with multiple unique allele sets (the combination of the alleles from the three genes) that we found as monophyletic. *Nothonotus microlepidus* and *N. sanguifluus* were not monophyletic, no haplotype sets were shared between the two species, and there was little resolution between these allele sets. Four of the five allele sets from Stones River *N. microlepidus* clustered together with significant support, but this clade included one allele set from Little River *N. microlepidus*. We also found a non-significantly supported clade containing one Big South Fork *N. sanguifluus* and eight of 11 Caney Fork *N. sanguifluus* allele sets.

The Bayesian analysis of the concatenated nuclear and mitochondrial dataset resulted in the most resolved tree (Fig. 5.4) and this tree was similar to previously hypothesized *Nothonotus* relationships (Etnier and Williams 1989; Keck and Near 2008; Near and Keck 2005; Wood 1996; Zorach 1967). *Nothonotus moorei* and *N. rubrus* share a MRCA with the *N. maculatus* species group instead of with *N. camurus* as in the cyt*b* tree or within the *N. maculatus* species group as in some of the nuclear gene trees. The *N. maculatus* species group is significantly supported and relationships within this clade are well resolved. We found *N. maculatus* as a significantly supported clade and sharing a MRCA with a clade containing *N. microlepidus* and *N. sanguifluus*. The Caney Fork and Big South Fork *N. sanguifluus* populations were each monophyletic, and the Big South Fork was supported with a significant Bayesian posterior. Thirteen of 16 *N. microlepidus* allele sets were monophyletic and the other three allele sets, from the Little River, Harpeth River, and Stones River, were distributed within a clade containing all of the *N. sanguifluus* allele sets from Buck Creek and the Rockcastle River.

The BEST analyses using the datasets with the fewest taxa, representing current taxonomy, and the most taxa, representing populations from each river as distinct taxa, were run for 160 and 200 million generations, respectively, and never converged. Plots of the likelihood scores by generation were still increasing at the end of each run. We ran the dataset identified in the methods as ‘Caney Fork and Big South Fork’, with an intermediate number of taxa, for just over 100 million generations and stopped this run because there was no sign of convergence. We abandon this method of delimiting *N. microlepidus* and *N. sanguifluus*.

The haplotype and allele networks revealed between drainage relationships that were not found in the genealogies, but there was still little signal extracted from MLL alleles. The cyt*b* haplotype network had geographic clusters corresponding to the geographic clusters found in the cyt*b* geneology and the Caney Fork *N. sanguifluus*, most of the Big South Fork *N. sanguifluus*, and *N. maculatus* clusters were connected to the remaining *N. sanguifluus* and *N. microlepidus* by relatively long branches (Fig. 5.5). The Buck Creek and Rockcastle *N. sanguifluus* haplotypes clustered with all of the *N. microlepidus* haplotypes and were as similar some of the *N. microlepidus* haplotypes as they were to other Buck Creek and Rockcastle *N. sanguifluus* haplotypes (Fig. 5.5). There was less geographic clustering of alleles in the S7 network, but most of the Caney Fork *N. sanguifluus* clustered together (Fig. 5.6). There was little variation in MLL for the *N. maculatus* species group and the allele network had no geographic signal (Fig. 5.7). There was little geographic clustering of RAG1 alleles and only the Harpeth and Little River populations of *N. microlepidus* had alleles that were not shared with *N. sanguifluus* (Fig. 5.8).

Meristic Analyses

We collected meristic data from 479 *N. sanguifluus* and 107 *N. microlepidus* sampled from 10 river systems (Table D.2). The data in each partition of each of the three analyses was normally distributed. Nineteen outliers were identified from box plots of each data partition and the case-wise z-scores of preliminary DA runs and we removed them, leaving data from 567 individuals in the subsequent analyses. The results of the Box's M tests for each run were significant, with $p<0.002$, indicating the variance matrices were not equal. However, the log determinants of the variance matrices were very similar within each run, suggesting Box's M was significant because of minimal differences in the matrices. All discriminant functions were significant with $p<0.001$. When the data was partitioned into two groups, *N. microlepidus* and *N. sanguifluus*, the cases were correctly classified 94.7% of the time, and fewer *N. sanguifluus* were classified as *N. microlepidus* than vise versa (Table 5.3). Adding the Caney Fork *N. sanguifluus* as a separate partition resulted in a decrease in correct classifications to 81.8% (Table 5.4). In the Caney Fork analysis, both *N. sanguifluus* groups were correctly classified more often than the *N. microlepidus* and nearly the same number of *N. microlepidus* were correctly classified as in the two taxa, current taxonomy analysis. The discriminant score plot from the Caney Fork analysis had three fairly distinct clusters (Fig. 5.9). When the data for Big South Fork *N. sanguifluus* was treated as a separate taxon, creating four groups, the percentage of correct classifications dropped to 77.4%, but *N. microlepidus* and Caney Fork *N. sanguifluus* are correctly classified more frequently than in the two previous analyses (Table 5.5). In the third analysis, Big South Fork *N. sanguifluus* and all other *N. sanguifluus* were correctly classified 79.3% and 35.8 % of the time, respectively. The 'all other *N. sanguifluus*' were more frequently classified as Caney Fork *N. sanguifluus*, whereas Caney Fork *N. sanguifluus* were most often misclassified as Big South Fork *N. sanguifluus*. The discriminant score plot from the third analysis had three fairly distinct clusters, as in the previous analysis, representing *N. microlepidus*, Caney Fork *N. sanguifluus*, and Big South Fork *N. sanguifluus*, but the fourth cluster representing all other *N. sanguifluus* was less distinct (Fig. 5.10). The all

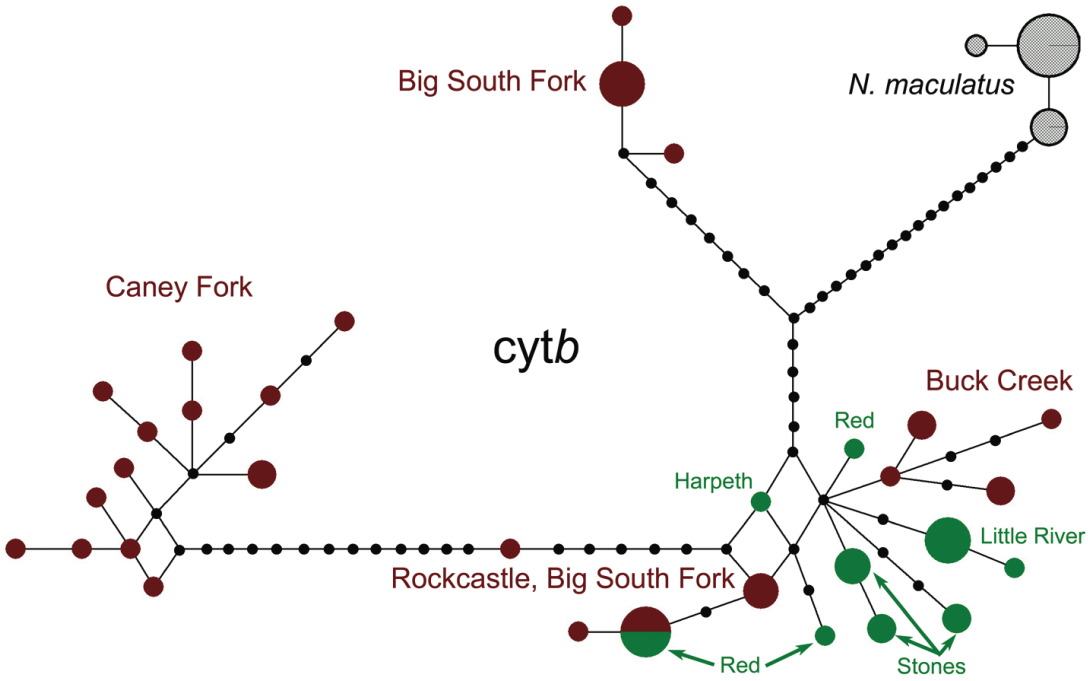

Fig. 5.5. Network resulting from median joining of *N. maculatus*, *N. microlepidus*, and *N. sanguifluus* cyt*b* haplotypes. Green indicates haplotypes isolated from *N. microlepidus*, red indicates haplotypes isolated from *N. sanguifluus*, and gray indicates *N. maculatus*. Circle diameter is positively associated with number of individuals observed with that haplotype. Black dots on branches indicate base pair changes not represented in observed haplotype. The geographic origin of the haplotypes is given in text of corresponding color near each circle where there is geographic clustering of haplotypes.

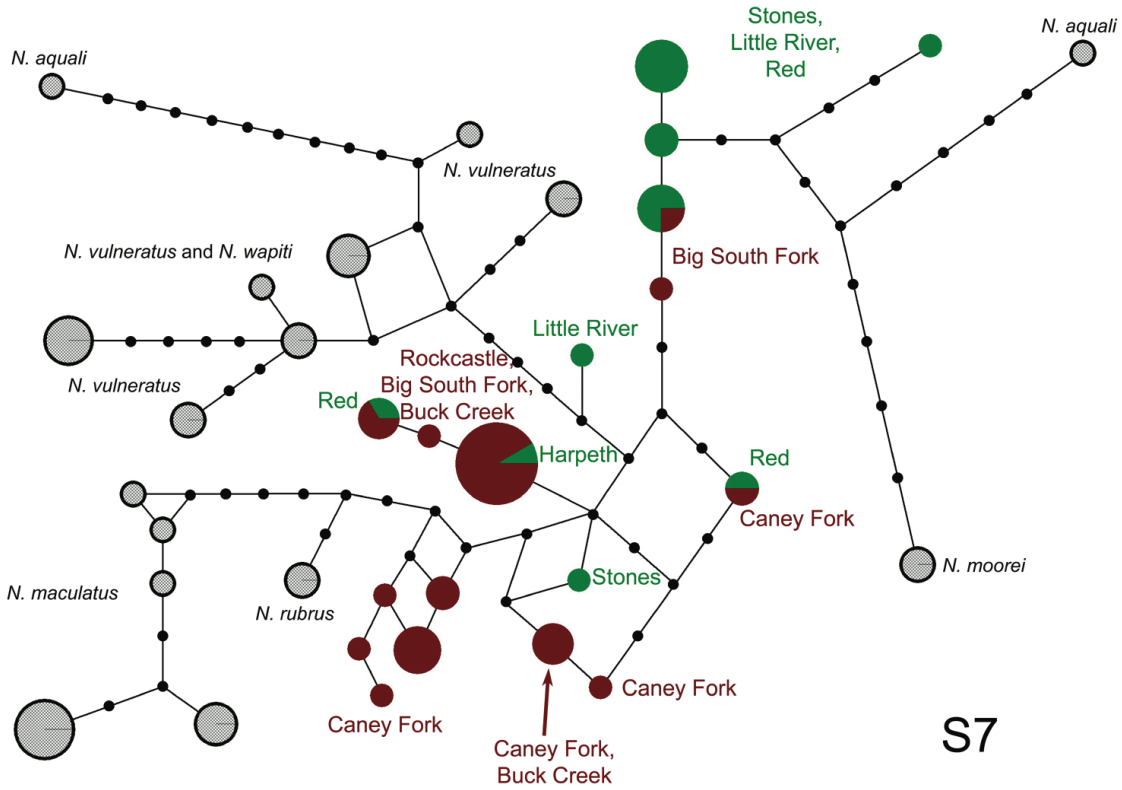

Fig. 5.6. Network resulting from median joining of all *N. maculatus* species group plus *N. moorei* and *N. rubrus* S7 alleles. Green indicates haplotypes isolated from *N. microlepidus*, red indicates haplotypes isolated from *N. sanguifluus*, and gray indicates other *N. maculatus* species group, with the species identified next to the circle. Circle diameter is positively associated with number of individuals observed with that allele. Black dots on branches indicate base pair changes not represented in observed allele. The geographic origin of the alleles is given in text of corresponding color near each circle where there is geographic clustering of alleles.

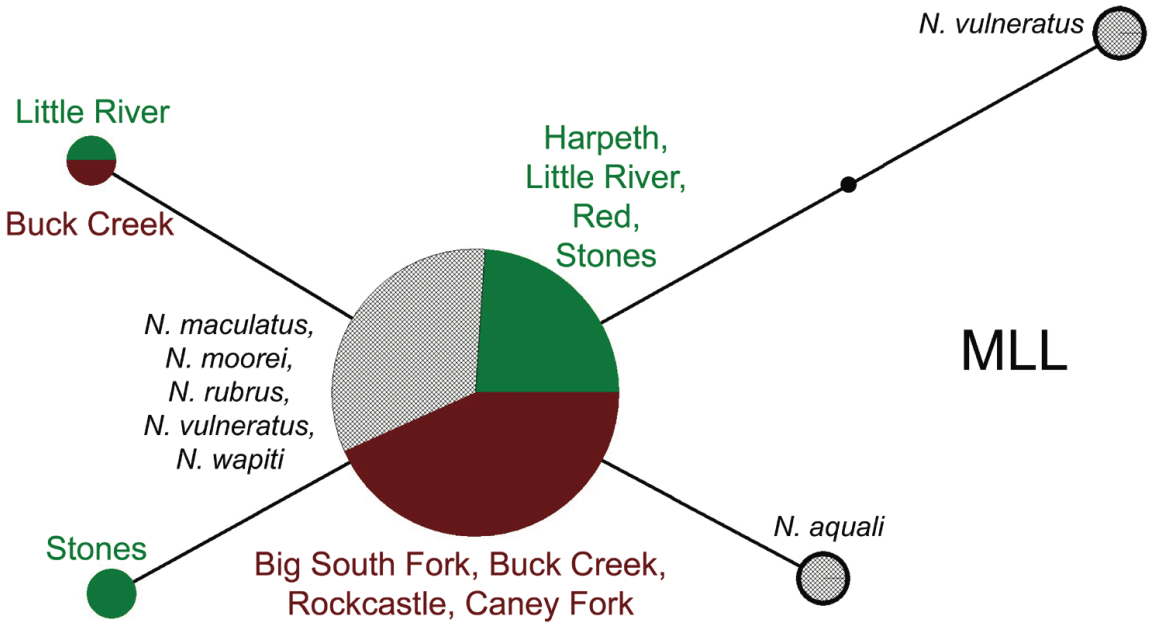

Fig. 5.7. Network resulting from median joining of all *N. maculatus* species group plus *N. moorei* and *N. rubrus* MLL alleles. Green indicates haplotypes isolated from *N. microlepidus*, red indicates haplotypes isolated from *N. sanguifluus*, and gray indicates other *N. maculatus* species group, with the species identified next to the circle. Circle diameter is positively associated with number of individuals observed with that allele. Black dots on branches indicate base pair changes not represented in observed allele. The geographic origin of the alleles is given in text of corresponding color near each circle where there is geographic clustering of alleles.

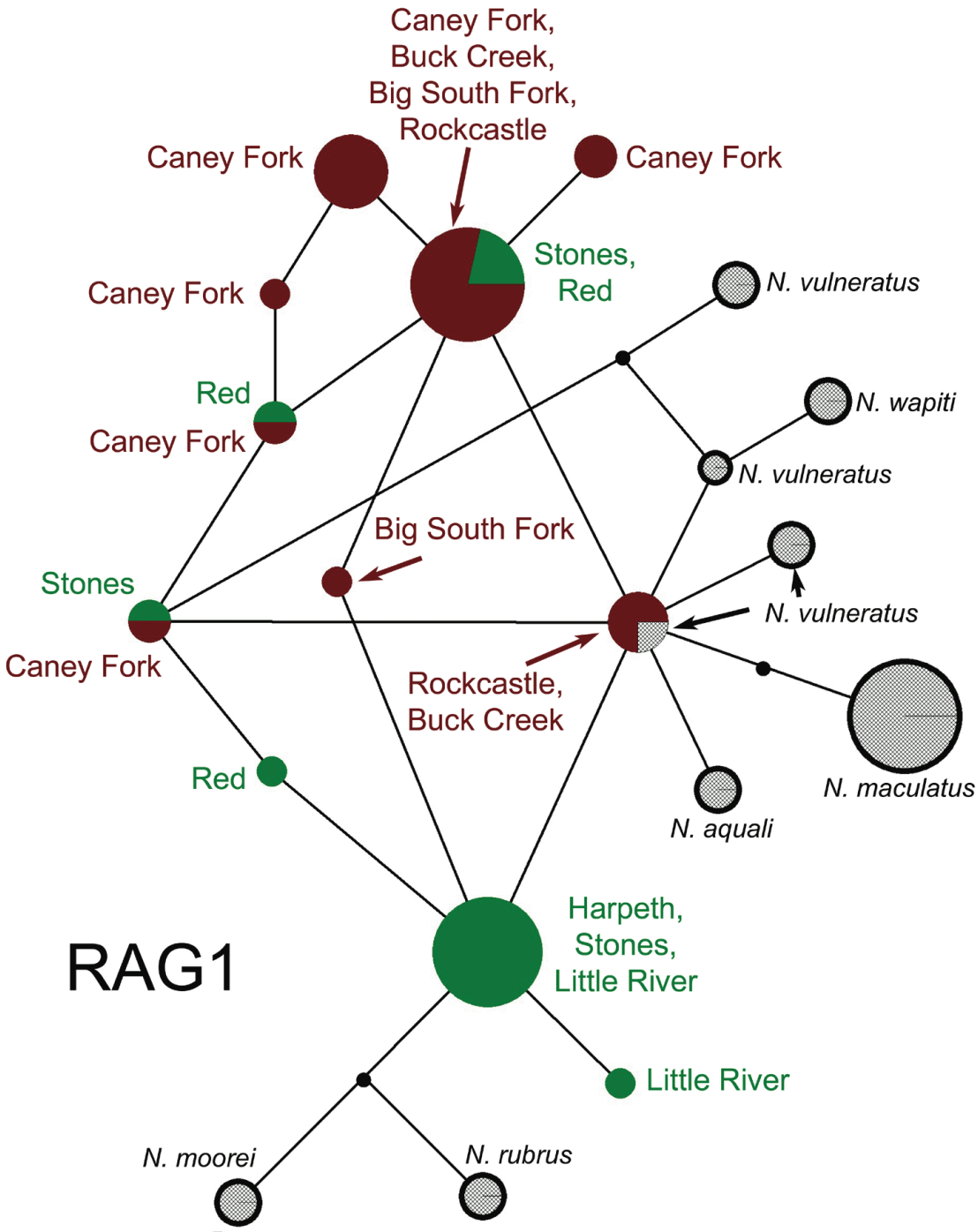

Fig. 5.8. Network resulting from median joining of all *N. maculatus* species group plus *N. moorei* and *N. rubrus* RAG1 alleles. Green indicates haplotypes isolated from *N. microlepidus*, red indicates haplotypes isolated from *N. sanguifluus*, and gray indicates other *N. maculatus* species group, with the species identified next to the circle. Circle diameter is positively associated with number of individuals observed with that allele. Black dots on branches indicate base pair changes not represented in observed allele. The geographic origin of the alleles is given in text of corresponding color near each circle where there is geographic clustering of alleles.

Table 5.3. Classification matrix from DA when there are two taxa, *N. microlepidus* and *N. sanguifluus*, based on current taxonomy. 94.7% of original grouped cases correctly classified.

			Predicted Group Membership		
		Group	*N. microlepidus*	*N. sanguifluus*	Total
Original	#	*N. microlepidus*	79	20	99
		N. sanguifluus	10	458	468
	%	*N. microlepidus*	79.8	20.2	100.0
		N. sanguifluus	2.1	97.9	100.0

Table 5.4. Classification matrix from DA when there are three taxa, *N. microlepidus*, *N. sanguifluus* from the Caney Fork River, and *N. sanguifluus* from all other rivers, based on genetic analyses. 81.8% of original grouped cases correctly classified.

			Predicted Group Membership			
		Group	*N. microlepidus*	*N. sanguifluus* All Others	*N. sanguifluus* Caney Fork	Total
Original	#	*N. microlepidus*	77	21	1	99
		N. sanguifluus – All Others	9	207	39	255
		N. sanguifluus – Caney Fork	3	30	180	213
	%	*N. microlepidus*	77.8	21.2	1.0	100.0
		N. sanguifluus – All Others	3.5	81.2	15.3	100.0
		N. sanguifluus – Caney Fork	1.4	14.1	84.5	100.0

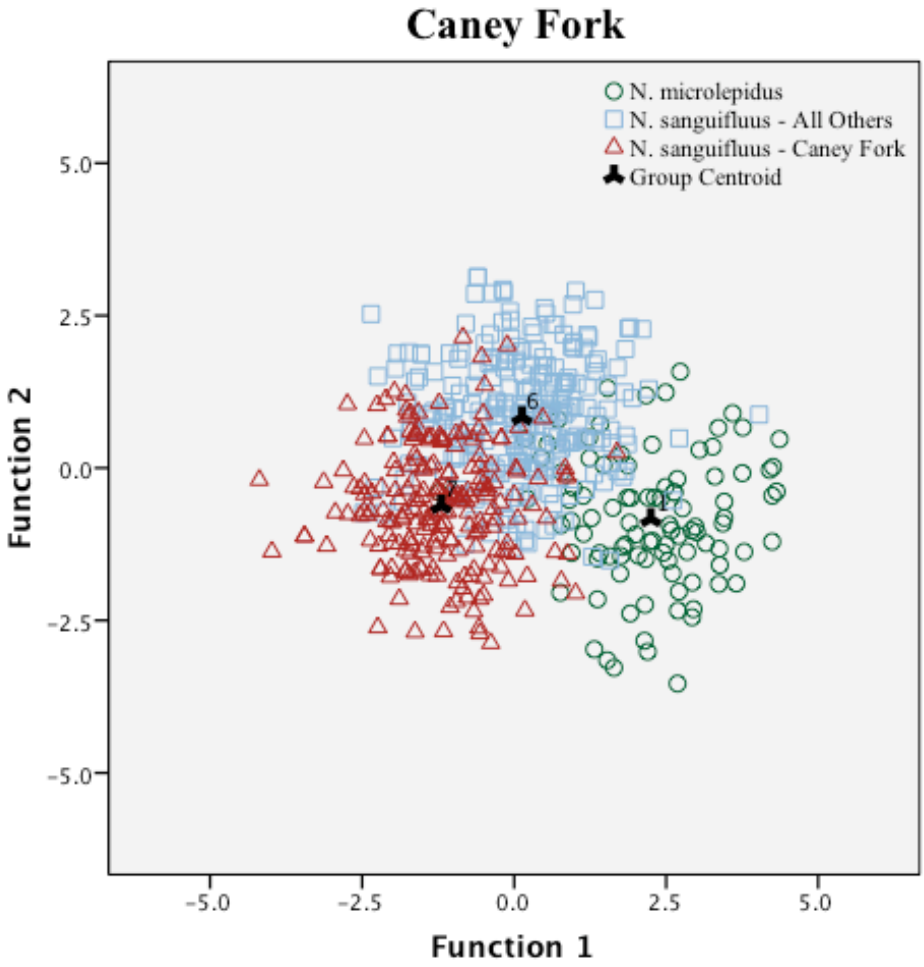

Fig. 5.9. Plot of discriminant scores for each individual by the first two significant discriminant functions when the taxa were *N. microlepidus*, *N. sanguifluus* from the Caney Fork, and *N. sanguifluus* from all other rivers.

Table 5.5. Classification matrix from DA when there are four taxa, *N. microlepidus*, *N. sanguifluus* from the Caney Fork River, *N. sanguifluus* from the Big South Fork River, and *N. sanguifluus* from all other rivers, based on genetic analyses. 77.4% of original grouped cases correctly classified.

			Predicted Group Membership				
		Group	*N. microlepidus*	*N. sanguifluus* Big South Fork	*N. sanguifluus* All Others	*N. sanguifluus* Caney Fork	Total
Original	#	*N. microlepidus*	81	15	1	2	99
		N. sanguifluus – Big South Fork	9	149	6	24	188
		N. sanguifluus – All Others	3	14	24	26	67
		N. sanguifluus – Caney Fork	3	18	7	185	213
	%	*N. microlepidus*	81.8	15.2	1.0	2.0	100.0
		N. sanguifluus – Big South Fork	4.8	79.3	3.2	12.8	100.0
		N. sanguifluus – All Others	4.5	20.9	35.8	38.8	100.0
		N. sanguifluus – Caney Fork	1.4	8.5	3.3	86.9	100.0

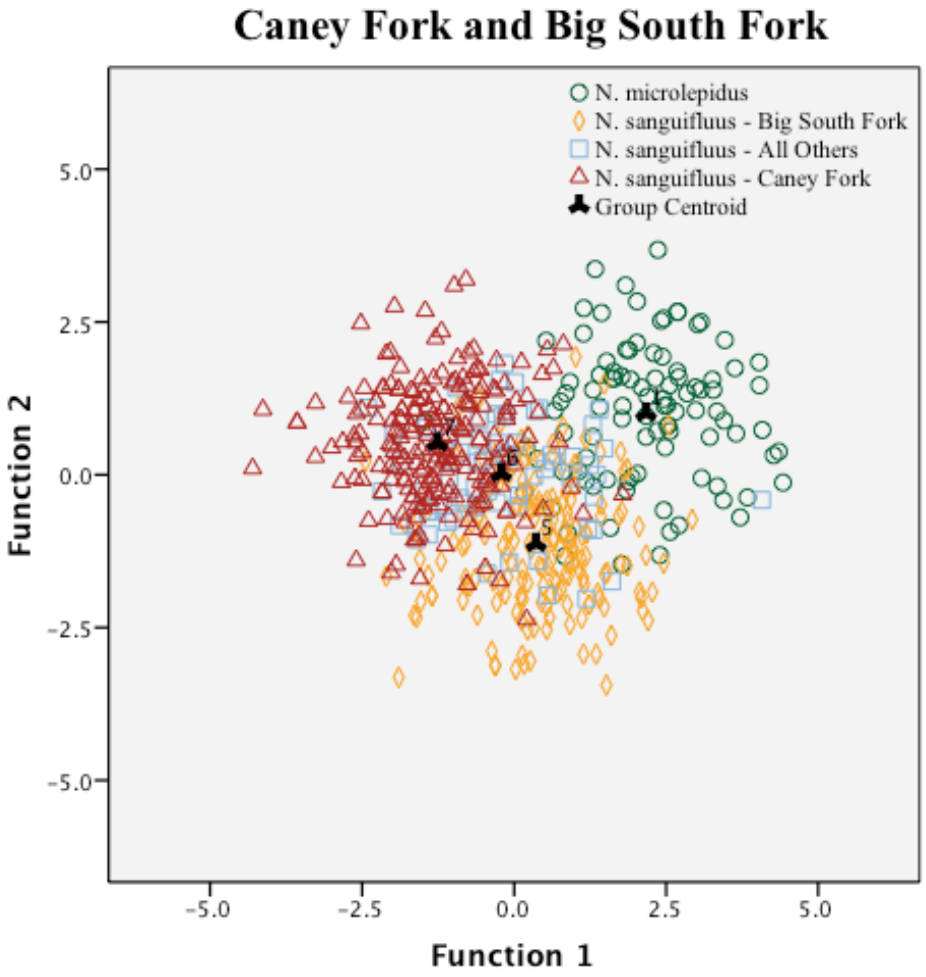

Fig. 5.10. Plot of discriminant scores for each individual by the first two significant discriminant functions when the taxa were *N. microlepidus*, *N. sanguifluus* from the Caney Fork, *N. sanguifluus* from the Big South Fork, and *N. sanguifluus* from all other rivers.

other *N. sanguifluus* cluster had its centroid in between the clusters of the other two *N. sanguifluus* groups and slightly more towards that of the *N. microlepidus* cluster. The fourth analysis, with three data partitions representing *N. sanguifluus* from the Caney Fork and Big South Fork as separate taxa and all remaining *N. sanguifluus* and all *N. microlepidus*, resulted in the second best correct classification of Caney Fork *N. sanguifluus*, but the poorest correct classification for Big South Fork *N. sanguifluus*, and only 68.1% of the remaining partition were correctly classified (Fig. 5.6). Partitioning the data by river drainage for both species resulted in non-normality of several of the partitions, inequality in most of the covariance matrices, and some partitions with fewer individuals than variables. Ignoring all of these violations and running DA resulted in only slightly lower correctly classified Big South Fork and Caney Fork *N. sanguifluus*, but all other populations were correctly classified no more than 35% of the time.

DISCUSSION

Patterns of divergence in eastern North American fish have primarily been studied at relatively large geographic scales (Hocutt and Wiley 1986; Mayden 1988; Near and Keck 2005; Near et al. 2001; Ray et al. 2006; Wiley and Mayden 1985), but many darter clades exhibit high levels of divergence within major river systems (Ceas and Page 1997; Hollingsworth and Near in press; Keck and Near 2008; Page et al. 1992; Page et al. 2003; Page and Near 2007) and this pattern of divergence has only recently been explored in one darter subclade (Hollingsworth and Near in press). The highly endemic darter fauna of the Cumberland River in Kentucky and Tennessee provides an opportunity to examine patterns of diversification across multiple darter subclades within a major river system. Here we show that darter diversification in the Cumberland River occurs at a small geographic scale in another darter subclade and that the responsible mechanisms have remained consistent over millions of years.

We recently identified a relationship between *N. microlepidus* and *N. sanguifluus* (Keck and Near 2008), two Cumberland River endemics, that indicated divergence within the Cumberland River. Previous studies hypothesized a relationship between *N. maculatus* and *N. sanguifluus*, but the position of *N. microlepidus* was either not resolved (Etnier and Williams 1989), found to be sister to a species outside of the Cumberland River (Wood 1996), or as sister to the *N. maculatus – N. sanguifluus* clade (Near and Keck 2005). The observed closer relationship of *N. maculatus* to *N. microlepidus* in a recent study (Keck and Near 2008) or *N. microlepidus* and some populations of *N. sanguifluus* in the cyt*b* tree in this study (Fig. 5.2) does not preclude a hypothesis of a *N. microlepidus – N. sanguifluus* sister relationship with divergence occurring within the Cumberland River. Several scenarios can account for this observed relationship, *e.g.*, peripheral isolation or short internodes between divergences, but none place the divergence of *N. microlepidus* and *N. sanguifluus*, exclusive of the Caney Fork population, as occurring outside the Cumberland River. The fact that the only scenario requiring Caney Fork *N. sanguifluus* divergence with only non-Cumberland River clades also requires the loss and subsequent re-evolution of *N. sanguifluus* like coloration supports the hypothesis of a *N. microlepidus – N. sanguifluus* clade, inclusive of all populations, sister to *N. maculatus*. The *N. microlepidus – N. sanguifluus* clade relationship indicates that divergence has occurred within the Cumberland River.

We were able to identify four distinct lineages in the *N. microlepidus – N. sanguifluus* clade using the results of the gene tree and morphometric analyses: 1) all *N. microlepidus*, 2) Caney Fork *N. sanguifluus*, 3) Big South Fork *N. sanguifluus*, and 4) all other *N. sanguifluus*. The current taxonomy recognizes only two lineages, *N. microlepidus* and *N. sanguifluus*, and DA is easily able to correctly classify individuals into their respective groups (~95% correct) using the meristic data (Table 5.3), but there are several lineages evident in the gene trees. The Caney Fork *N. sanguifluus* were monophyletic in the cyt*b* (Fig. 5.2) and concatenated trees (Fig. 5.4), and the cyt*b* haplotype and S7 allele networks had clusters of the Caney Fork *N. sanguifluus* haplotypes and alleles (Figs. 5.5 and 5.6). In all of the DA analyses, the Caney Fork *N. sanguifluus* individuals were correctly classified more often than any other group (Tables 5.3, 5.4, 5.5, and 5.6). The lowest correct classification percentage was 84.5% (Table 5.4) and that was still higher than the correct classification for any other group. The Big South Fork *N. sanguifluus* were monophyletic and had a significant Bayesian posterior probability in the concatenated mitochondrial/nuclear tree (Fig. 5.4), but shared one cyt*b* haplotype with the Rockcastle River *N. sanguifluus* (Fig. 5.2 and 5.5) and had no resolution in the nuclear gene trees (Fig. 5.3), indicating the isolation of the Big South Fork population is either more recent or there is limited gene flow. The results of DA indicate that Big South Fork *N. sanguifluus* were correctly classified nearly as often as *N. microlepidus* were (~80%)(Table 5.5), indicating that the grouping was identifiable using the meristic characters. The next clade suggested by the genetic data contained all *N. microlepidus*, and *N. sanguifluus* from the Rockcastle River and Buck Creek. The *N. microlepidus* and two *N. sanguifluus* populations were paraphyletic in the cyt*b* tree (Fig. 5.2), concatenated tree (Fig. 5.4), and sharing alleles in all of the nuclear trees (Fig. 5.3), indicating that these populations were derived from the same ancestors. There was some within river system association of cyt*b* haplotypes in the network, but relationships between them were still unresolved (Fig. 5.5). However, partitioning the meristic data with this grouping of *N. microlepidus* and *N. sanguifluus* reduced the correct classification of all other groups, and itself was correctly classified less than 70% of the time (Table 5.6). The poor classification values when using this grouping, along with the distinct color differences between *N. microlepidus* and *N. sanguifluus*, indicate that the remaining *N. sanguifluus* and *N. microlepidus* are distinct lineages. The four lineages described above indicate that the current taxonomy does not fully describe the diversity of *N. sanguifluus-N. microlepidus* within the Cumberland River.

The addition of distinct lineages to the *N. microlepidus – N. sanguifluus* clade does not bring the number to near the level observed in the barcheek clade (Hollingsworth and Near in press). The disparity in the number of distinct lineages between the barcheeks and *Nothonotus* may be influenced by several factors or any combination of them. The first and most obvious possibility is missing data restricts identification of more groups. The Buck Creek and Rockcastle River *N. sanguifluus* do show some genetic differentiation (Fig. 5.2 and 5.4), but partitioning the meristic data into more groups resulted in violating all assumptions for DA. The fact that the 'all others' group, in the DA with Big South Fork and Caney Fork *N. sanguifluus* as separate groups, had extremely poor classification results (Table 5.5), indicates that this grouping is probably incorrect. The likely extirpation of several of the *N. sanguifluus* populations

Table 5.6. Classification matrix from DA when there are three taxa, *N. sanguifluus* from the Caney Fork River, *N. sanguifluus* from the Big South Fork River, and all remaining *N. microlepidus* and *N. sanguifluus*, based on genetic analyses. 77.8% of original grouped cases correctly classified.

			Predicted Group Membership			
		Group	*N. microlepidus* and *N. sanguifluus*	*N. sanguifluus* Big South Fork	*N. sanguifluus* Caney Fork	Total
Original	#	*N. microlepidus* and *N. sanguifluus*	113	24	29	166
		N. sanguifluus – Big South Fork	22	144	22	188
		N. sanguifluus – Caney Fork	11	18	184	213
	%	*N. microlepidus* and *N. sanguifluus*	68.1	14.5	17.5	100.0
		N. sanguifluus – Big South Fork	11.7	76.6	11.7	100.0
		N. sanguifluus – Caney Fork	5.2	8.5	86.4	100.0

inhibits expanding the meristic or genetic datasets, thus identification is currently limited to the above lineages. The *N. microlepidus – N. sanguifluus* clade is a much younger clade than the barcheek clade, 1.4 million and 16.6 million years respectively, and this means that mechanisms of diversification have influenced the barcheek clade more than 10 times as long as the *N. microlepidus – N. sanguifluus* clade. Additionally, the younger age of the *Nothonotus* clade and the fact that both *Nothonotus* are often three to five times more abundant than barcheeks (author's pers. obs.) make it more likely to find retained ancestral polymorphism despite isolation. Regardless of the mechanism, the result would be an underestimation of the number of *Nothonotus* lineages in the Cumberland River.

Microendemism in *Nothonotus* is consistent with the barcheek pattern (Hollingsworth and Near in press) and the responsible mechanisms are persistent and ancient influences in the Cumberland River. Four lineages in the *N. microlepidus – N. sanguifluus* clade were revealed in the genetic and morphometric analyses, but the young age and potential extirpated populations inhibit the identification of more clades. These lineages indicate substantial diversification has occurred at geographic scales much smaller than those usually considered (Hocutt and Wiley 1986; Mayden 1988; Near and Keck 2005; Near et al. 2001; Ray et al. 2006; Wiley and Mayden 1985). The fact that the same pattern has occurred in clades with very disparate ages indicates that the mechanisms involved have persisted in the Cumberland River over significant evolutionary time.

Microendemism in the Cumberland River has implications for evolutionary study and conservation planning for darters and other animal clades. The ancient and apparently continuous influence of the same mechanisms is evidence that diversification in the Cumberland River darters, and likely other animal clades, is not due to major geologic 'events' such as river captures and glaciations. More comparative studies across clades may reveal what those mechanisms are, or what characteristics make a lineage more prone to these influences. Two studies of Cumberland River darters have revealed a small geographic scale of diversification and discovered multiple distinct lineages previously unrecognized indicating conservation planning should incorporate exploratory studies at relatively fine scales. For instance, *N. sanguifluus* occurs in nearly half of the Cumberland River, but this cannot be considered a management unit because there are distinct lineages in nearly every tributary sampled in this study, including some that may warrant recognition as separate species. Additionally, several populations that may have represented distinct taxa have been extirpated from the Cumberland River, including *N. sanguifluus* from three tributaries (this study) and *N. camurus* from at least two tributaries (Etnier and Starnes 1993; Zorach 1972). Identification of distinct lineages is particularly important for North American aquatic fauna because this fauna is experiencing an extinction rate five times greater than terrestrial animals (Ricciardi and Rasmussen 1999) and the number of imperiled North American freshwater and diadromous fish increased by 92% in just under two decades (Jelks et al. 2008).

LITERATURE CITED

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APPENDICES

APPENDIX A
Supplemental material for Chapter 2

Table A.1. List of records included with putative identification, museum catalog number or citation, number of individuals in the lot, general locality, and year of collection. Dates noted with an asterisk are records of a cross taken from the same locality at different times. Under Catalog #:H et al., 1988 = Hubbs et al., 1988; H & L, 1961a and H & L, 1961b = Hubbs & Laritz, 1961a and Hubbs & Laritz, 1961b.

Hybrid Identification	Catalog #	Ind.	Locality and Date
A. beanii X *A. meridiana*	UAIC 4425.36	1	Buttahatchie Creek, Lowndes Co., MS, 1972
A. beanii X *A. meridiana*	TU 81666	1	Alabama River, Wilcox Co., AL, 1973
E. artesiae X *E. stigmaeum*	UAIC 1593.01	1	New River, Winston Co., AL, 1965
E. blennioides X *E. caeruleum*	UT 91.3960	1	Wabash River, Vigo Co., IN, 1990
E. blennioides X *E. spectabile*	INHS 101259	1	Vermilion River, Champaign Co., IL , 2006
E. caeruleum X *E. fragi*	INHS 36053	1	Strawberry River, Sharp Co., AR, 1995
E. caeruleum X *E. lawrencei*	UMMZ 165475	1	Clear Creek, Rockcastle Co., KY, 1953
E. caeruleum X *E. lawrencei*	SIUC 27005	1	Marrowbone Creek, Cumberland Co., KY, 1996
E. caeruleum X *E. spectabile*	UMMZ 167125	1	Bear Creek, Monroe Co., MI, 1952
E. caeruleum X *E. spectabile*	INHS 32695	2	Little Lake Creek, Wayne Co., MO, 1994
E. caeruleum X *E. spectabile*	INHS 60783	9	Muskingum River, Licking Co., OH, 1991
E. caeruleum X *E. spectabile*	OSUM 95359	1	Sweeny Run, Scioto Co., OH, 2001
E. caeruleum X *E. spectabile*	OSUM 98537	1	Stoney Run, Clermont Co., OH, 2002
E. caeruleum X *E. spectabile*	OSUM 38932	1	Cole Camp Creek, Benton Co., MO, 1976
E. caeruleum X *E. spectabile*	OSUM 98721	1	Rock Run, Clark Co., OH, 2002
E. caeruleum X *E. spectabile*	OSUM 94531	3	Lisbon Fork, Clark Co., OH, 1995
E. caeruleum X *E. spectabile*	OSUM 93062	1	Massies Creek, Greene Co., OH, 2000
E. caeruleum X *E. spectabile*	OSUM 90779	3	Little Miami River, Clark Co., OH, 1993*
E. caeruleum X *E. spectabile*	OSUM 101135	1	Little Miami River, Clark Co., OH, 1993*
E. caeruleum X *E. spectabile*	OSUM 90961	1	Little Miami River, Clark Co., OH, 1999*
E. caeruleum X *E. spectabile*	OSUM 90994	1	Little Miami River, Clark Co., OH, 1999*
E. caeruleum X *E. spectabile*	OSUM 96567	1	Little Miami River, Clark Co., OH, 2000*
E. caeruleum X *E. spectabile*	OSUM 24381	2	Blacklick Creek, Franklin Co., OH, 1972
E. caeruleum X *E. spectabile*	OSUM 90117	2	Bales Ditch, Madison Co., OH, 1999
E. caeruleum X *E. spectabile*	OSUM 90995	1	Lisbon Fork, Clark Co., OH, 1999
E. caeruleum X *E. spectabile*	OSUM 30203	1	Fall Creek, Marion Co., IN, 1942
E. caeruleum X *E. spectabile*	OSUM 100278	1	Big Darby Creek, Logan Co., OH, 1983
E. caeruleum X *E. spectabile*	CU 45502	1	Kentucky River, Madison Co., KY, 1953
E. caeruleum X *E. spectabile*	SIUC 63615	7	Licking River, Bath Co., KY, 2004
E. caeruleum X *N. rufilineatus*	INHS 83934	1	Cedar Creek, Franklin Co., AL, 1978
E. caeruleum X *P. caprodes*	OSUM 28356	1	Shankatank Creek, Rush Co., IN, 1941
E. caeruleum X *P. maculata*	OSUM 45661	2	Wellington Creek, Lorain Co., OH, 1977
E. chlorosoma X *E. gracile*	UMMZ 161011	1	Forked Deer River, Crockett Co., T, 1949
E. chlorosoma X *E. stigmaeum*	TU 23609	1	McGee's Creek, Walthall Co., MS, 1960
E. collis X *E. olmstedi*	UT 91.3501	1	Sawneys Creek, Fairfield Co., SC, 1988
E. collis X *E. olmstedi*	NCSM 28488	3	Walker Branch, Mecklenburg Co., NC, 1997
E. corona X *E. nigripinne*	UAIC 5042.14	12	South Fork Buffalo R., Lawrence Co., TN, 1976
E. corona X *E. nigripinne*	INHS 61800	3	Horse Creek, Hardin Co., TN, 1986
E. corona X *E. nigripinne*	INHS 63867	2	Buffalo River, Wayne Co., TN, 1988
E. corona X *E. nigripinne*	INHS 63925	6	Shakerag Creek, Wayne Co., TN, 1988
E. corona X *E. nigripinne*	INHS 45548	5	Indian Creek, Hardin Co., TN, 1998
E. crossopterum X *E. nigripinne*	INHS 40892	5	Buffalo River, Lewis Co., TN, 1997
E. crossopterum X *E. nigripinne*	INHS 40893	2	Buffalo River, Lewis Co., TN, 1996
E. crossopterum X *E. nigripinne*	INHS 61745	23	Duck River, Maury Co., TN, 1986
E. crossopterum X *E. nigripinne*	INHS 62775	5	Duck River, Maury Co., TN, 1987
E. crossopterum X *E. nigripinne*	INHS 62813	7	Duck River, Bedford Co., TN, 1987

Table A.1. Continued

Hybrid Identification	Catalog #	Ind.	Locality and Date
E. crossopterum X *E. nigripinne*	INHS 62829	13	Duck River, Bedford Co., TN, 1987
E. crossopterum X *E. nigripinne*	INHS 62843	18	Buffalo River, Lawrence Co., TN, 1987
E. crossopterum X *E. nigripinne*	INHS 63918	5	Duck River, Bedford Co., TN, 1988
E. duryi X *E. simoterum*	UAIC 13574.07	1	Lick Fork Paint Rock River, Jackson Co., AL, 2002
E. flavum X *E. planasaxatile*	UT 91.5929	1	Flat Creek, Maury Co., TN, 1997
E. forbsei X *E. nigripinne*	UMMZ 121052	6	Duck River, Bedford Co., TN, 1937
E. forbsei X *E. nigripinne*	UMMZ 121122	1	Duck River, Bedford Co., TN, 1937
E. forbsei X *E. nigripinne*	UMMZ 121137	13	Duck River, Bedford Co., TN, 1937
E. forbesi X *E. nigripinne*	INHS 58379	5	Duck River, Coffee Co., TN, 1987
E. forbesi X *E. nigripinne*	INHS 62790	5	Duck River, Coffee Co., TN, 1987
E. forbesi X *E. nigripinne*	INHS 62792	8	Duck River, Bedford Co., TN, 1987
E. forbesi X *E. nigripinne*	INHS 64782	18	Duck River, Coffee Co., TN, 1989
E. forbesi X *E. nigripinne*	INHS 75761	4	Duck River, Coffee Co., TN, 1965
E. gracile X *P. maculata*	Page, 1976	1	Dismal Creek, Fayette Co., IL, 1964
E. kennicotti X *E. squamiceps*	Page, 1974	1	Big Creek, Hardin Co., IL, 1971
E. lepidum X *P. caprodes*	H et al., 1988	1	Guadalupe River, Kerr Co., TX, 1986
E. lepidum X *E. spectabile*	H et al., 1988	2	Guadalupe River, Kerr Co., TX, 1986
E. punctulatum X *E. spectabile*	OSUM 48572	1	Pomme de Terre River, Hickory Co., MO, 1979
E. radiosum X E. spectabile	KU 12930	79	Blue River, Johnston Co., OK, 1968*
E. radiosum X *E. spectabile*	UMMZ 162608	2	Blue River, Johnston Co., OK, 1949*
E. radiosum X *E. spectabile*	ANSP 153621	18	Blue River, Johnston Co., OK, 1984
E. radiosum X *E. spectabile*	OK 46291	4	Bridge Creek, Hempstead Co., AR, 1990
E. radiosum X *E. spectabile*	INHS 81065	13	Blue River, Johnston Co., OK, 1978
E. radiosum X *E. spectabile*	CU 76815	16	Blue River, Pontotoc Co., OK, 1977
E. radiosum X *E. spectabile*	SIUC 37729	1	Blue River, Johnston Co., OK, 1999
E. ramseyi X *E. rupestre*	UAIC 2040.1	1	Beaver Creek, Wilcox Co., AL, 1966
E. raneyi X *P. nigrofasciata*	TU 29455	1	Yellow River, Gwinnett Co., GA, 1962
E. spectabile X *E. whipplei*	UMMZ 162607	1	Sand Creek, Osage Co., OK, 1950
E. spectabile X *P. caprodes*	KU 15183	1	Rock Creek, Douglas Co., KS, 1973
E. spectabile X *P. sciera*	H & L, 1961a	1	San Marcos River, Hays Co., TX, 1960
E. stigmaeum X *E. tallapoosae*	UAIC 10334.04	1	Snake Creek, Cleburne Co., AL, 1992
E. zonale X *P. caprodes*	UT 91.2643	1	Paint Rock River, Madison Co., AL, 1983
N. acuticeps X *N. chlorobranchius*	UT 91.4091	1	Nolichucky River, Unicoi Co., TN, 1991
N. camurus X *N. chlorobranchius*	UT 91.4040	11	Nolichucky River, Washington Co., TN, 1991
N. camurus X *N. microlepidus*	UT 91.4057	1	Red River, Robertson Co., TN, 1991
N. camurus X *N. rufilineatus*	UT 91.1290	1	Nolichucky River, Cocke Co., TN, 1975*
N. camurus X *N. rufilineatus*	UT 91.3331	1	Nolichucky River, Hamblen Co., TN, 1987*
N. camurus X *N. rufilineatus*	UT 91.3629	1	North Fork Holston River, Sullivan Co., TN, 1989
N. camurus X *N. rufilineatus*	UT 91.4042	1	Red River, Cocke Co., TN , 1991
N. camurus X *N. rufilineatus*	UT 91.1255	1	Nolichucky River, Cocke Co., TN, 1976
N. camurus X *N. rufilineatus*	UT 91.3642	1	Little River, Blount Co., TN, 1988*
N. camurus X *N. rufilineatus*	UT 91.2350	1	Little River, Blount Co., TN, 1979*
N. camurus X *N. rufilineatus*	UT 91.2668	1	Clinch River, Hancock Co., TN, 1983
N. camurus X *N. rufilineatus*	UMMZ 104112	1	Clear Creek, Anderson Co., TN, 1937
N. camurus X *N. tippecanoe*	INHS 83898	1	Big South Fork River, Scott Co., TN, 1978
N. camurus X *N. tippecanoe*	OSUM 500	1	Big Darby Creek, Pickaway Co., OH, 1939*
N. camurus X *N. tippecanoe*	OSUM 64686	1	Big Darby Creek, Pickaway Co., OH, 1985*
N. chlorobranchius X *N. rufilineatus*	UAIC 8970.03	2	Citico Creek, Monroe Co., TN, 1980

Table A.1. Continued

Hybrid Identification	Catalog #	Ind.	Locality and Date
N. chlorobranchius X *N. rufilineatus*	UT 91.825a	17	Little Pigeon River, Sevier Co., TN, 1973*
N. chlorobranchius X *N. rufilineatus*	UT 91.825b	2	Little Pigeon River, Sevier Co., TN, 1989*
N. chlorobranchius X *N. rufilineatus*	UT 91.1961	2	Tabcat Creek, Blount Co., TN, 1979
N. chlorobranchius X *N. rufilineatus*	UT 91.2334	1	Watauga River, Johnson Co., TN, 1978
N. chlorobranchius X *N. rufilineatus*	UT 91.4526	1	Little Pigeon River, Sevier Co., TN, 1994
N. chlorobranchius X *N. rufilineatus*	UT 91.5253	8	Abrams Creek, Blount Co., TN, 1997*
N. chlorobranchius X *N. rufilineatus*	UT 91.5420a	9	Abrams Creek, Blount Co., TN, 1998*
N. chlorobranchius X *N. rufilineatus*	UT 91.5420b	3	Abrams Creek, Blount Co., TN, 2000*
N. chlorobranchius X *N. rufilineatus*	UT 91.4320	1	Little Tennessee River, Swain Co., NC, 1992
N. chlorobranchius X *N. rufilineatus*	INHS 68660	1	French Broad R., Transylvania Co., NC, 1985
N. microlepidus X *N. rufilineatus*	INHS 79306	1	Turnbull Creek, Cheatham Co., TN, 1978
N. rufilineatus X *N. vulneratus*	UT 91.3333	1	Nolichucky River, Hamblen Co., TN, 1987
N. rufilineatus X *N. vulneratus*	UMMZ 129470	1	Abrams Creek, Blount Co., TN, 1937
N. rufilineatus X *P. caprodes*	UT 91.3715	1	Beech Creek, Hawkins Co., TN, 1990
N. rufilineatus X *P. evides*	UAIC 11539.22	1	Cedar Creek, Franklin Co., AL, 1996
N. rufilineatus X *P. squamata*	UT 91.3386	1	Pigeon River, Cocke Co., TN, 1988
N. rufilineatus X *P. squamata*	UT 91.4736	1	Emory River, Morgan Co., TN, 1995
P. aurantiaca X *P. caprodes*	UT 91.4131	1	Emory River, Morgan Co., TN, 1991
P. aurolineata X *P. lenticula*	TU 69116	1	Cahaba River, Bibb Co., AL, 1971
P. burtoni X *P. sciera*	UT 91.6306	1	Holston River, Hawkins Co., TN, 1997
P. caprodes X *P. copelandi*	ROM 38014	1	Lake Erie, ON, Canada, 1979
P. caprodes X *P. copelandi*	UMMZ 65802	1	Au Sable River, Iosco Co., MI, 1924
P. caprodes X *P. copelandi*	UMMZ 174669	1	Pine River, Alcona Co., MI, 1957
P. caprodes X *P. evides*	UT 91.4743	1	Elk River, Giles Co., TN, 1995
P. caprodes X *P. macrocephala*	CU 68506	1	Green River, Monroe Co., KY, 1955
P. caprodes X *P. macrocephala*	INHS 39198	1	French Creek, Crawford Co., PA, 1996
P. caprodes X *P. maculata*	UMMZ 60201	3	Huron River, Livingston Co., MI, 1920
P. caprodes X *P. maculata*	UMMZ 60439	1	Thames River, ON, Canada, 1923
P. caprodes X *P. maculata*	UMMZ 63014	4	Tippecanoe Lake, IN, 1904
P. caprodes X *P. maculata*	UMMZ 64224	1	Strawberry Lake, MI, 1931
P. caprodes X *P. maculata*	UMMZ 65759	2	Au Sable River, Alcona Co., MI, 1924*
P. caprodes X *P. maculata*	UMMZ 65779	1	Au Sable River, Iosco Co., MI, 1924*
P. caprodes X *P. maculata*	UMMZ 65817	5	Au Sable River, Iosco Co., MI, 1924*
P. caprodes X *P. maculata*	UMMZ 66743	5	Au Sable River, Iosco Co., MI, 1924*
P. caprodes X *P. maculata*	UMMZ 73141	2	Au Sable River, Iosco Co., MI, 1925*
P. caprodes X *P. maculata*	UMMZ 74768	1	Nocque Bay Lake, Marinette, WI, 1926
P. caprodes X *P. maculata*	UMMZ 80823	1	Huron River, MI, 1927
P. caprodes X *P. maculata*	UMMZ 104462	1	Manistee River, Wexford Co., MI, 1936
P. caprodes X *P. maculata*	UMMZ 112010	1	Huron River., Washtenaw Co., MI, 1935
P. caprodes X *P. maculata*	UMMZ 145652	1	Big Kinkaid Creek, Jackson Co., IL, 1939

Table A.1. Continued

Hybrid Identification	Catalog #	Ind.	Locality and Date
P. caprodes X *P. maculata*	UMMZ 160800	1	Thunder Bay River, Alpena Co., MI, 1950
P. caprodes X *P. maculata*	UMMZ 167303	2	Huron River, Livingston Co., MI, 1954
P. caprodes X *P. maculata*	UMMZ 213168	1	Tippecanoe Lake, Kosciusko Co., IN, 1985
P. caprodes X *P. maculata*	UMMZ 213737	1	Cheboygan River, Cheboygan Co., MI, 1986
P. caprodes X *P. maculata*	INHS 40662	1	St. Croix River, Chisago Co., MN, 1996
P. caprodes X *P. maculata*	INHS 67985	1	Little Missouri River, Picke Co., AR, 1983
P. caprodes X *P. maculata*	INHS 69039	1	Tippecanoe River, Kosciusko Co., IN, 1985
P. caprodes X *P. maculata*	OSUM 3691	1	Auglaize River, Auglaize Co., OH, 1940
P. caprodes X *P. maculata*	OSUM 3398	1	Muddy Creek, Ottawa Co., OH, 2008
P. caprodes X *P. maculata*	OSUM 4117	1	Unnamed, Auglaize Co., OH, 1941
P. caprodes X *P. maculata*	OSUM 9669	1	Stillwater River, Darke Co., OH, 1930
P. caprodes X *P. maculata*	Univ. Tulsa 831	1	Mountain Fork, McCurtain Co., OK, 1966
P. caprodes X *P. maculata*	CU 44013	1	Cuba Lake, Allegany Co., NY, 1937
P. caprodes X *P. maculata*	SIUC 40224	1	Middle Fork Kentucky River, Leslie Co., KY, 1995
P. caprodes X *P. phoxocephala*	UT 91.3528	1	Duck River, Humphrys Co., TN, 1988*
P. caprodes X *P. phoxocephala*	UT 91.831	1	Duck River, Humphrys Co., TN, 1973*
P. caprodes X *P. phoxocephala*	UT 91.2397	1	Bear Creek, Colbert Co., AL, 1982
P. caprodes X *P. phoxocephala*	KU 12978	1	Four Mile Creek, Butler Co., KS, 1963
P. caprodes X *P. phoxocephala*	KU 26365	1	Bloody Creek, Chase Co., KS, 1997
P. caprodes X *P. phoxocephala*	OSUM 53683	1	Cuivre River, Lincoln Co., MO, 1979
P. caprodes X *P. sciera*	TNHC 123	1	San Marcos River, Caldwell Co., TX, 1949
P. caprodes X *P. sciera*	UAIC 8580.34	6	Shoal Creek, Lauderdale Co., AL, 1985
P. caprodes X *P. sciera*	H & L, 1961b	2	San Gabriel River, Williamson Co., TX, 1961
P. caprodes X *P. shumardi*	UT 91.6063	1	Ohio River, Gallia Co., OH, 2000
P. caprodes X *P. shumardi*	UT 91.2686	2	Sequatchie River, Marion Co., TN, 1981
P. caprodes X *P. shumardi*	UMMZ 65850	1	Au Sable River, Iosco Co., MI, 1924*
P. caprodes X *P. shumardi*	UMMZ 73131	2	Au Sable River, Iosco Co., MI, 1925*
P. caprodes X *P. shumardi*	UMMZ 71987	1	Mississippi River, Pierce Co., WI, 1926
P. caprodes X *P. shumardi*	UMMZ 78017	1	St. Croix River, St. Croix Co., WI, 1928
P. caprodes X *P. williamsi*	UT 91.2549	1	Little River, Blount Co., TN, 1983*
P. caprodes X *P. williamsi*	UT 91.2674	1	Little River, Blount Co., TN, 1983*
P. caprodes X *P. williamsi*	UT 91.2859	1	Little River, Blount Co., TN, 1985*
P. caprodes X *P. williamsi*	UT 91.3282	1	Little River, Blount Co., TN, 1987*
P. copelandi X *P. maculata*	ROM 70952	1	Lake Erie, ON, Canada, 1997
P. kathae X *P. smithvanizi*	UAIC 13546.08	1	Wind Creek, Tallapoosa Co., AL, 2002
P. maculata X *P. phoxocephala*	OSUM 14019	2	Kaskasia River, Moultrie Co., IL, 1965
P. maculata X *P. phoxocephala*	Page, 1976	1	Elk Fork, Monroe Co., MO, 1973
P. nigrofasciata X *P. sciera*	TU 40887	1	Pearl River, Washington Co., LA, 1965*
P. nigrofasciata X *P. sciera*	TU 40888	1	Pearl River, Washington Co., LA, 1965*
P. nigrofasciata X *P. sciera*	TU 39380	2	Pearl River, Washington Co., LA, 1965*
P. nigrofasciata X *P. sciera*	TU 39726	6	Pearl River, Washington Co., LA, 1966*
P. nigrofasciata X *P. sciera*	TU 40000	2	Pearl River, Washington Co., LA, 1966*
P. nigrofasciata X *P. sciera*	TU 40150	1	Strong River, Simpson Co., LA, 1966*
P. nigrofasciata X *P. sciera*	TU 41891	1	Pearl River, Washington Co., LA, 1966*
P. nigrofasciata X *P. sciera*	TU 42122	1	Pearl River, Washington Co., LA, 1966*
P. nigrofasciata X *P. sciera*	TU 42162	2	Pearl River, Washington Co., LA, 1966*
P. nigrofasciata X *P. sciera*	TU 42201	2	Pearl River, Washington Co., LA, 1966*
P. nigrofasciata X *P. sciera*	TU 42443	2	Pearl River, Washington Co., LA, 1966*
P. nigrofasciata X *P. sciera*	TU 42706	6	Pearl River, Washington Co., LA, 1966*
P. nigrofasciata X *P. sciera*	TU 42904	2	Pearl River, Washington Co., LA, 1967*
P. nigrofasciata X *P. sciera*	TU 43273	2	Pearl River, Washington Co., LA, 1967*

Table A.1. Continued

Hybrid Identification	Catalog #	Ind.	Locality and Date
P. nigrofasciata X *P. sciera*	TU 43829	1	Pearl River, Washington Co., LA, 1967*
P. nigrofasciata X *P. sciera*	TU 44250	1	Pearl River, Washington Co., LA, 1967*
P. nigrofasciata X *P. sciera*	TU 49073	1	Pearl River, Washington Co., LA, 1967*
P. nigrofasciata X *P. sciera*	TU 49103	1	Pearl River, Washington Co., LA, 1967*
P. nigrofasciata X *P. sciera*	TU 49796	1	Pearl River, Washington Co., LA, 1968*
P. nigrofasciata X *P. sciera*	TU 50380	2	Pearl River, Washington Co., LA, 1968*
P. nigrofasciata X *P. sciera*	TU 50543	3	Pearl River, Washington Co., LA, 1968*
P. nigrofasciata X *P. sciera*	TU 50753	1	Pearl River, Washington Co., LA, 1968*
P. nigrofasciata X *P. sciera*	TU 50861	3	Pearl River, Washington Co., LA, 1968*
P. nigrofasciata X *P. sciera*	TU 56006	3	Pearl River, Washington Co., LA, 1969*
P. nigrofasciata X *P. sciera*	TU 56133	2	Pearl River, Washington Co., LA, 1969*
P. nigrofasciata X *P. sciera*	TU 57440	1	Pearl River, Washington Co., LA, 1969*
P. nigrofasciata X *P. sciera*	TU 59529	1	Pearl River, Washington Co., LA, 1969*
P. nigrofasciata X *P. sciera*	TU 61792	1	Pearl River, St. Tammany Co., LA, 1969*
P. nigrofasciata X *P. sciera*	TU 60533	1	Strong River, Simpson Co., LA, 1969*
P. nigrofasciata X *P. sciera*	TU 71123	3	Pearl River, Washington Co., LA, 1971*
P. nigrofasciata X *P. sciera*	TU 75748	1	Pearl River, Washington Co., LA, 1972*
P. nigrofasciata X *P. sciera*	TU 75793	1	Pearl River, Washington Co., LA, 1972*
P. nigrofasciata X *P. sciera*	TU 79395	1	Pearl River, Washington Co., LA, 1972*
P. nigrofasciata X *P. sciera*	TU 81437	1	Pearl River, St. Tammany Co., LA, 1972*
P. nigrofasciata X *P. sciera*	TU 81728	3	Pearl River, Washington Co., LA, 1973*
P. nigrofasciata X *P. sciera*	TU 85917	1	Leaf River, Jones Co., MS, 1973
P. nigrofasciata X *P. sciera*	TU 86709	2	Pearl River, St. Tammany Co., LA, 1973*
P. nigrofasciata X *P. sciera*	TU 86728	1	Pearl River, St. Tammany Co., LA, 1973*
P. nigrofasciata X *P. sciera*	TU 86515	2	Pearl River, Washington Co., LA, 1974*
P. nigrofasciata X *P. sciera*	TU 93694	11	Pearl River, Washington Co., LA, 1975*
P. nigrofasciata X *P. sciera*	TU 93954	3	Pearl River, Washington Co., LA, 1975*
P. nigrofasciata X *P. sciera*	TU 96999	3	Pearl River, Washington Co., LA, 1976*
P. nigrofasciata X *P. sciera*	TU 97612	1	Strong River, Simpson Co., MS, 1976*
P. nigrofasciata X *P. sciera*	TU 99829	1	Pearl River, St. Tammany Co., LA, 1976*
P. nigrofasciata X *P. sciera*	TU 101657	2	Pearl River, Washington Co., LA, 1977*
P. nigrofasciata X *P. sciera*	TU 105472	1	Pearl River, Washington Co., LA, 1977*
P. nigrofasciata X *P. sciera*	TU 105744	1	Pearl River, Washington Co., LA, 1978*
P. nigrofasciata X *P. sciera*	TU 106359	1	Pearl River, Washington Co., LA, 1978*
P. nigrofasciata X *P. sciera*	TU 109969	1	Pearl River, Washington Co., LA, 1978*
P. nigrofasciata X *P. sciera*	TU 115516	1	Pearl River, Lawrence Co., MS, 1979*
P. nigrofasciata X *P. sciera*	TU 116351	3	Pearl River, Washington Co., LA, 1980*
P. nigrofasciata X *P. sciera*	TU 116704	1	Tangipahoa River, Pike Co., MS, 1980
P. nigrofasciata X *P. sciera*	TU 118401	1	Pearl River, Lawrence Co., MS, 1980*
P. nigrofasciata X *P. sciera*	TU 120288	2	Pearl River, Washington Co., LA, 1981*
P. nigrofasciata X *P. sciera*	TU 124579	1	Pearl River, Pearl River Co., MS, 1982*
P. nigrofasciata X *P. sciera*	TU 128536	1	Pearl River, Lawrence Co., LA, 1982*
P. nigrofasciata X *P. sciera*	TU 128768	1	Pearl River, Pearl River Co., MS, 1983*
P. nigrofasciata X *P. sciera*	TU 148240	1	Pearl River, Pearl River Co., MS , 1987*
P. nigrofasciata X *P. sciera*	TU 159214	1	Pearl River, Pearl River Co., MS, 1990*
P. nigrofasciata X *P. sciera*	TU 177199	1	Pearl River, St. Tammany Co., LA, 1995*
P. nigrofasciata X *P. sciera*	TU 178410	1	Pearl River, Pearl River Co., MS, 1996*
P. nigrofasciata X *P. sciera*	TU 181513	2	Pearl River, St. Tammany Co., LA, 1996*
P. nigrofasciata X *P. sciera*	TU 193491	1	Pearl River, Lawrence Co., MS, 2001*
P. nigrofasciata X *P. shumardi*	UT 91.3726	1	Shoal Creek, St. Clair Co., AL, 1990
P. notogramma X *P. peltata*	UR 2462	1	South Anna River, Hanover Co., VA, 1964
P. notogramma X *P. peltata*	UR 2463	1	Rivanna River, Fluvanna Co., VA, 1967

Table A.1. Continued

Hybrid Identification	Catalog #	Ind.	Locality and Date
P. notogramma X *P. peltata*	RC-REJ 888	1	Appomattox River, Appomattox Co., VA, 1979
P. oxyrhyncha X *P. roanoka*	VPI 2610	1	New River, Pulaski Co., VA, 1971
P. palmaris X *P. smithvanizi*	UAIC 10557.16	1	Tallapoosa River, Chambers Co., AL, 1988
P. phoxocephala X *P. sciera*	UMMZ 81384	1	Patoka River, IN, 1927
P. phoxocephala X *P. sciera*	OSUM 90081	2	Paint Creek, Ross Co., OH , 1997
P. phoxocephala X *P. shumardi*	UT 91.4523	1	Duck River, Humphreys Co., TN, 1993
P. sciera X *P. sipsi*	UAIC 13298.19	1	Sipsey Fork, Winston Co., AL, 2001
P. shumardi X *P. vigil*	UT 91.2666	1	Duck River, Humphreys Co., TN, 1983

APPENDIX B
Supplemental material for Chapter 3

Table B.1. Specimens sampled, geographic localities, and catalog numbers.

Species	Code	YFTC	Locality	Lat (N)	Long (W)
Perca flavescens	PflaA	261	Lake Andrusia, Beltrami Co., MN	47 27 23	94 39 13
Sander vitreus	SvitA	312	Mississippi R., Rock Island Co., IL	41 30 46	90 35 22
Crystallaria asprella	CaspB	686	Cahaba R., Bibb Co., AL	32 56 52	87 08 26
Ammocrypta pellucida	ApelA	104	Embarrass R., Cumberland Co., IL	39 15 01	88 10 27
Percina roanoka	ProaA	76	Blackwater R., Franklin Co., VA	37 03 14	79 52 56
P. evides	PeviL	1134	Black R., Jackson Co., WI	44 15 45	90 52 14
P. caprodes	PcapD	396	Big Piney Fork, Sharp Co., AR	36 04 50	91 36 39
Etheostoma cinereum	EcinA	689	Rockcastle R., Rockcastle Co., KY	37 17 33	84 13 14
E. blennioides	EbleA	756	West Fork Pond R., Christian Co., KY	37 01 52	87 24 21
E. flabellare	EflaB	1438	Middle Fork of the Vermillion R., Vermillion Co., IL	40 14 06	87 46 18
E. virgatum	EvirF	781	Clear Cr., Rockcastle Co., KY	37 28 10	84 17 07
Nothonotus acuticeps	NacuA	2205	Nolichucky R., Greene Co., TN	36 06 00	83 02 51
N. acuticeps	NacuB	5659	Nolichucky R., Greene Co., TN	36 06 00	83 02 51
N. acuticeps	NacuC	5660	Nolichucky R., Greene Co., TN	36 06 00	83 02 51
N. acuticeps	NacuI	5666	Nolichucky R., Greene Co., TN	36 06 00	83 02 51
N. aquali	NaquA	68	Buffalo R., Lewis Co., TN	35 27 44	87 32 10
N. aquali	NaquB	2562	Duck R., Bedford Co., TN	35 33 12	86 34 57
N. aquali	NaquD	69	Buffalo R., Lewis Co., TN	35 27 44	87 32 10
N. bellus	NblmA	754	Goose Cr., Russell Co., KY	37 05 56	85 03 05
N. bellus	NblmB	4865	Long Cr., Macon Co., TN	36 35 01	85 55 47
N. bellus	NblmD	6451	Petty's Fork of Russell Cr., Adair Co., KY	37 05 57	85 20 16
N. bellus	NblmF	6453	Petty's Fork of Russell Cr., Adair Co., KY	37 05 57	85 20 16
N. bellus	NblmK	6306	Middle Fork Drakes Cr., Allen Co., KY	36 41 31	86 20 53
N. camurus	NcamA	1203	Middle Fork of the Vermillion R., Vermillion Co., IL	40 14 06	87 46 18
N. camurus	NcamC	2582	Clinch R., Claiborne Co., TN	36 25 48	83 23 49
N. camurus	NcamI	5249	Brimstone Cr., Scott Co., TN	36 18 17	84 30 18
N. camurus	NcamM	3309	Little R., Blount Co., TN	35 45 56	83 51 23
N. camurus	NcamP	5669	Nolichucky R., Greene Co., TN	36 06 00	83 02 51
N. camurus	NcamZ	6492	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59
N. camurus	NcamAA	5640	Holston R., Jefferson Co., TN	36 06 02	83 40 09
N. camurus	NcamAK	7063	Big South Fork, Scott Co., TN	36 32 49	84 39 56
N. camurus	NcamAM	7017	Rockcastle R., Laurel/Rockcastle Co. line, KY	37 16 46	84 12 35
N. camurus	NcamAO	6519	Sinking Cr., Laurel Co., KY	37 05 23	84 12 12
N. chlorobranchius	NchbA	2120	Burningtown Cr., Macon Co., NC	35 15 23	83 28 20
N. chlorobranchius	NchbC	3094	Little Pigeon R., Sevier Co., TN	35 44 21	83 24 59
N. chlorobranchius	NchbD	5967	North Fork Mills R., Henderson Co., NC	35 24 22	82 38 39
N. chlorobranchius	NchbE	5968	North Fork Mills R., Henderson Co., NC	35 24 22	82 38 39
N. chlorobranchius	NchbI	6803	North Indian Cr., Unicoi Co., TN	36 08 36	82 25 42
N. chuckwachatte	NckwA	2275	Tallapoosa R., Haralson Co., GA	33 51 49	85 12 47
N. chuckwachatte	NckwB	5609	Cane Cr., Cleburne Co., AL	33 43 31	85 29 27
N. chuckwachatte	NckwC	5610	Cane Cr., Cleburne Co., AL	33 43 31	85 29 27
N. chuckwachatte	NckwD	5611	Cane Cr., Cleburne Co., AL	33 43 31	85 29 27
N. denoncourti	NdenA	2585	Clinch R., Claiborne Co., TN	36 25 48	83 23 49
N. denoncourti	NdenB	4108	Sequatchie R., Sequatchie Co., TN	35 04 45	85 35 35
N. douglasi	NdouA	2116	Sipsey Fork, Winston Co., AL	34 17 07	87 23 57
N. douglasi	NdouB	5540	Borden Cr., Lawrence Co., AL	34 19 46	87 22 37
N. douglasi	NdouC	5567	Sipsey Fork, Winston Co., AL	34 17 07	87 23 57
N. douglasi	NdouD	5568	Sipsey Fork, Winston Co., AL	34 17 07	87 23 57
N. douglasi	NdouF	5539	Borden Cr., Lawrence Co., AL	34 19 46	87 22 37
N. etowahae	NetoA	2272	Etowah R., Lumpkin Co., GA	34 32 06	84 03 48
N. etowahae	NetoC	4089	Shoal Cr., Dawson Co., GA	34 25 57	84 07 45
N. jordani	NjorA	985	Conasauga R., Polk Co., TN.	35 00 35	84 43 55
N. jordani	NjorB	2366	Conasauga R., Polk Co., TN.	35 00 09	84 46 44
N. jordani	NjorD	2363	Conasauga R., Polk Co., TN.	35 00 09	84 46 44
N. jordani	NjorE	2364	Conasauga R., Polk Co., TN.	35 00 09	84 46 44
N. jordani	NjorF	2365	Conasauga R., Polk Co., TN.	35 00 09	84 46 44
N. juliae	NjulA	897	Buffalo R., Searcy Co., AR	35 59 08	92 44 41

Table B.1. Continued.

Species	Code	YFTC	Locality	Lat (N)	Long (W)
N. juliae	NjulC	2957	Buffalo R., Searcy Co., AR	35 59 08	92 44 41
N. juliae	NjulD	2958	Buffalo R., Searcy Co., AR	35 59 08	92 44 41
N. juliae	NjulE	2959	Buffalo R., Searcy Co., AR	35 59 08	92 44 41
N. maculatus	NmacA	446	Green R., Green Co., KY	37 15 13	85 30 10
N. microlepidus	NmclA	2249	Harpeth R., Cheatham Co., TN	36 07 23	87 05 57
N. microlepidus	NmclB	5487	East Fork Stones R., Rutherford Co., TN	35 52 57	86 16 22
N. microlepidus	NmclC	5488	East Fork Stones R., Rutherford Co., TN	35 52 57	86 16 22
N. microlepidus	NmclD	5489	East Fork Stones R., Rutherford Co., TN	35 52 57	86 16 22
N. moorei	NmorA	2896	Middle Fork Little Red R., Van Buren Co., AR	35 39 11	92 18 56
N. moorei	NmorC	2898	Middle Fork Little Red R., Van Buren Co., AR	35 39 11	92 18 56
N. moorei	NmorL	2884	Middle Fork Little Red R., Van Buren Co., AR	35 31 20	92 26 26
N. rubrus	NrubB	4912	Bayou Pierre, Copiah Co., MS	31 52 12	90 29 52
N. rubrus	NrubC	4913	Bayou Pierre, Copiah Co., MS	32 00 10	90 41 22
N. rubrus	NrubD	4914	Foster's Cr., Copiah Co., MS	31 56 18	90 37 17
N. rufilineatus	NrufH	2493	Horse Cr., Hardin Co., TN	35 10 50	88 12 35
N. rufilineatus	NrufI	2494	Horse Cr., Hardin Co., TN	35 10 50	88 12 35
N. rufilineatus	NrufAM	5747	Elk R., Moore/Franklin Co., TN	35 09 49	86 19 06
N. rufilineatus	NrufAV	770	Grinders Cr., Lewis Co., TN	35 27 44	87 32 10
N. rufilineatus	NrufBB	6595	Duck R., Coffee Co., TN	35 29 07	86 07 18
N. rufilineatus	NrufBK	771	Grinders Cr., Lewis Co., TN	35 27 44	87 32 10
N. rufilineatus	NrufCQ	6594	Duck R., Coffee Co., TN	35 29 07	86 07 18
N. sanguifluus	NsgfA	940	Rockcastle R., Rockcastle Co., KY	37 17 33	84 13 14
N. sanguifluus	NsgfB	2444	Scott Cr., Warren Co., TN	35 34 17	85 42 43
N. sanguifluus	NsgfG	2472	Collins R., Warren Co., TN	35 44 46	85 41 54
N. sanguifluus	NsgfH	3128	Cane Cr., VanBuren Co., TN	35 46 57	85 24 16
N. sanguifluus	NsgfI	3129	Cane Cr., VanBuren Co., TN	35 46 57	85 24 16
N. sanguifluus	NsgfO	6504	Buck Cr., Pulaski Co., KY	37 09 05	84 26 18
N. sanguifluus	NsgfQ	6506	Buck Cr., Pulaski Co., KY	37 09 05	84 26 18
N. sanguifluus	NsgfT	7070	Big South Fork, Scott Co., TN	36 32 49	84 39 56
N. sanguifluus	NsgfV	7068	Big South Fork, Scott Co., TN	36 32 49	84 39 56
N. sanguifluus	NsgfZ	7022	Rockcastle R., Laurel/Rockcastle Co. line, KY	37 16 46	84 12 35
N. tippecanoe	NtipA	6424	Green R., Green Co., KY	37 15 59	85 34 52
N. tippecanoe	NtipB	7084	Big South Fork, Scott Co., TN	36 32 49	84 39 56
N. tippecanoe	NtipF	813	Licking R., Pendleton Co., KY	38 47 22	84 22 03
N. tippecanoe	NtipG	459	South Fork Kentucky R., Clay Co., KY	37 16 24	83 38 47
N. vulneratus	NvulA	2203	Taccoa R., Fannin Co., GA	34 45 51	84 14 56
N. vulneratus	NvulB	2204	Oconoluftee R., Jackson Co., NC	35 28 07	83 19 28
N. vulneratus	NvulE	2591	Clinch R., Claiborne Co., TN	36 25 48	83 23 49
N. vulneratus	NvulG	460	Daddys Cr., Cumberland Co., TN	36 03 32	84 47 32
N. vulneratus	NvulH	5677	Nolichucky R., Greene Co., TN	36 06 00	83 02 51
N. vulneratus	NvulI	5776	Little R., Blount Co., TN	35 47 16	83 52 55
N. wapiti	NwapB	4097	Elk R., Giles/Lincoln Co., TN	35 04 56	86 49 60
N. wapiti	NwapE	4100	Elk R., Giles/Lincoln Co., TN	35 04 56	86 49 60
N. wapiti	NwapF	4101	Elk R., Giles/Lincoln Co., TN	35 04 56	86 49 60

Table B.2. Coding of discrete morphological characters for *Nothonotus* species. Character 1:Dark lines on side of body, (0) absent, (1) present; character 2: color on breast, (0) absent, (1) present; character 3: scales on nape, (0) absent, (1) present; character 4: scales on opercle, (0) absent, (1) present; character 5: dark bands on fin margins, (0) absent, (1) present; character 6: red spots on side of body, (0) absent, (1) present; character 7: round halos on side of body, (0) absent, (1) present; character 8: sexual dimorphism in median fin coloration, (0) absent, (1) present; character 9: scales on upper cheek, (0) absent, (1) present; character 10: scales on belly, (0) absent, (1) present; character 11: modal number of vertebrae, (0) ≥ 37, (1) ≤ 36; character 12: bright color on anal fin of males, (0) absent, (1) present.

Species	1	2	3	4	5	6	7	8	9	10	11	12
Nothonotus juliae	1	0	0	0	0	0	0	0	0	0	1	0
Nothonotus denoncourti	0	1	1	0	0	0	0	1	0	1	1	0
Nothonotus tippecanoe	0	1	1	0	0	0	0	1	0	1	1	0
Nothonotus rufilineatus	1	1	1	0	1	1	0	1	0	0	0	0
Nothonotus acuticeps	1	1	1	1	0	0	0	0	0	0	0	0
Nothonotus chuckwachatte	0	1	1	1	1	1	0	1	0	0	0	0
Nothonotus etowahae	0	1	1	1	1	0	0	1	0	0	0	0
Nothonotus douglasi	0	1	1	0	1	0	0	1	0	0	0	0
Nothonotus jordani	0	1	1	1	1	1	0	1	0	0	0	0
Nothonotus moorei	1	1	1	0	1	0	0	1	1	1	1	0
Nothonotus rubrus	1	1	1	0	1	1	0	1	1	1	1	0
Nothonotus chlorobranchius	1	1	1	0	1	1	0	0	0	0	0	0
Nothonotus camurus	1	1	1	0	1	1	0	0	0	0	0	0
Nothonotus bellus	1	1	1	0	1	1	0	0	0	0	0	0
Nothonotus vulneratus	1	1	1	0	1	1	1	1	1	0	0	1
Nothonotus aquali	1	1	1	0	0	1	1	1	1	0	0	0
Nothonotus sanguifluus	1	1	1	0	0	1	1	1	1	0	0	0
Nothonotus maculatus	1	1	1	0	0	1	1	1	1	0	0	0
Nothonotus microlepidus	1	1	1	0	1	1	1	1	1	0	0	0
Nothonotus wapiti	1	1	1	0	1	0	0	1	1	0	0	1
outgroup	0	0	0	0	0	0	0	0	0	0	0	0

Table B.3. Uncorrected pairwise DNA sequence divergence observed within *Nothonotus* species sampled with three or more specimens. The minimum (Min) and maximum (Max) intraspecific divergences observed at the mitochondrial cytochrome *b* gene (cytb) and the nuclear encoded S7 intron 1 is reported for each species.

Species (number sampled)	Min cytb	Max cytb	Mean cytb	Min S7	Max S7	Mean S7
Nothonotus acuticeps (4)	0.00088	0.00439	0.00263	0	0	0
Nothonotus aquali (3)	0.00088	0.00526	0.00351	0.01149	0.03643	0.02366
Nothonotus bellus (5)	0.00175	0.00965	0.00614	0	0.00766	0.00249
Nothonotus camurus (10)	0	0.00965	0.00590	0	0.01156	0.00236
Nothonotus chlorobranchius (5)	0.00263	0.03070	0.01771	0	0.01924	0.00862
Nothonotus chuckwachatte (4)	0	0.00439	0.00219	0	0.00383	0.00191
Nothonotus douglasi (5)	0.00088	0.00351	0.00207	0	0.02323	0.01291
Nothonotus jordani (5)	0	0.01670	0.00773	0	0.00382	0.00193
Nothonotus juliae (4)	0	0.00175	0.00088	0	0.00386	0.00193
Nothonotus microlepidus (4)	0	0.00439	0.00307	0	0.01530	0.00510
Nothonotus moorei (3)	0	0.00176	0.00117	0	0	0
Nothonotus rubrus (3)	0	0	0	0	0	0
Nothonotus rufilineatus (7)	0.00088	0.02018	0.01295	0	0.02536	0.01195
Nothonotus sanguifluus (10)	0	0.02281	0.01542	0	0.00958	0.00414
Nothonotus tippecanoe (4)	0	0.00351	0.00175	0	0	0
Nothonotus vulneratus (6)	0.00351	0.02807	0.01749	0.00191	0.01153	0.00500
Nothonotus wapiti (3)	0	0.00088	0.00058	0	0.00574	0.00319

Table B.4. Maximum likelihood ancestral reconstruction reported as the proportional likelihood probabilities (*P*) of discrete characters at the *Nothonotus* most recent common ancestor node.

Character	*P* absent	*P* present	Number of changes
1. Dark lines on side of body	0.780	0.220	0
2. Color on breast	0.999	0.001	1
3. Scales on nape	0.999	0.001	1
4. Scales on opercle	0.999	0.001	2
5. Dark bands on fin margins	0.857	0.143	2
6. Red spots on flanks	0.980	0.020	3
7. Red spots surrounded by red halos	0.999	0.001	2
8. Dimorphism in median fins	0.863	0.137	2
9. Scales on upper cheek	0.999	0.001	1
10. Scales on belly	0.999	0.001	2
11. Modal number of vertebrae ≥ 37	0.987	0.013	3
12. Bright color on anal fin (males)	0.999	0.001	1

APPENDIX C
Supplemental material for Chapter 4

Table C.1. Specimens sampled, geographic localities and catalogue numbers. **YFTC**= Yale Fish Tissue Collection, **INHS**= Illinois Natural History Survey, **NCSM**= North Carolina State Museum, **TU**= Tulane University, **UT**= University of Tennessee Research Collection of Fishes, **YPM**= Yale Peabody Museum of Natural History, and **n.v.**= no voucher taken.

Species	Catalogue	Locality	Lat (N)	Long (W)	Voucher
Perca flavescens	YFTC 261	Lake Andrusia, Beltrami Co., MN	47 27 23	94 39 13	INHS 39508
Sander vitreus	YFTC 312	Mississippi R., Rock Island Co., IL	41 30 46	90 35 22	n.v.
Percina roanoka	YFTC 76	Blackwater R., Franklin Co., VA	37 03 14	79 52 56	INHS 64359
Crystallaria asprella	YFTC 686	Cahaba R., Cumberland Co., IL	32 56 52	87 08 26	n.v.
Ammocrypta pellucida	YFTC 104	Embarrass R., Cumberland Co., IL	39 15 01	88 10 27	n.v.
Etheostoma cinereum	YFTC 689	Rockcastle R., Rockcastle Co., KY	37 17 33	84 13 14	n.v.
E. blennioides	YFTC 756	West Fk. Pond R., Christian Co., KY	37 01 52	87 24 21	INHS 32700
E. virgatum	YFTC 781	Clear Cr., Rockcastle Co., KY	37 28 10	84 17 07	INHS 37939
Nothonotus acuticeps	YFTC 5659	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7042
	YFTC 5660	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7042
N. aquali	YFTC 68	Buffalo R., Lewis Co., TN	35 27 44	87 32 10	n.v.
	YFTC 2562	Duck R., Bedford Co., TN	35 33 12	86 34 57	UT 91.6607
N. bellus	YFTC 754	Goose Cr., Russell Co., KY	37 05 56	87 32 10	INHS 63870
	YFTC 6451	Petty's Fk. of Russell Cr., Adair Co., KY	37 05 57	85 20 16	UT 91.7331
N. camurus	YFTC 833	Middle Fk. Kentucky R., Leslie Co., KY	37 13 16	83 22 34	INHS 42972
	YFTC 1203	Middle Fk. Vermillion R., Vermillion Co., IL	40 14 06	87 46 18	n.v.
	YFTC 1204	Middle Fk. Vermillion R., Vermillion Co., IL	40 14 06	87 46 18	n.v.
	YFTC 2578	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.6526
	YFTC 2582	Clinch R., Claiborne Co., TN	36 25 48	83 23 49	UT 91.6529
	YFTC 2583	Clinch R., Claiborne Co., TN	36 25 48	83 23 49	UT 91.6529
	YFTC 2584	Clinch R., Claiborne Co., TN	36 25 48	83 23 49	UT 91.6529
	YFTC 3255	Holston R., Grainger Co., TN	36 25 49	83 23 49	UT 91.6747
	YFTC 3256	Holston R., Grainger Co., TN	36 25 49	83 23 49	UT 91.6747
	YFTC 3257	Holston R., Grainger Co., TN	36 25 49	83 23 49	UT 91.6747
	YFTC 3309	Little R., Blount Co., TN	35 45 56	83 51 23	UT 91.6789
	YFTC 5249	Brimstone Cr., Scott Co., TN	36 18 17	84 30 18	UT 91.7131
	YFTC 5250	Brimstone Cr., Scott Co., TN	36 18 17	84 30 18	UT 91.7131
	YFTC 5251	Brimstone Cr., Scott Co., TN	36 18 17	84 30 18	UT 91.7131
	YFTC 5252	Brimstone Cr., Scott Co., TN	36 18 17	84 30 18	UT 91.7131
	YFTC 5253	Brimstone Cr., Scott Co., TN	36 18 17	84 30 18	UT 91.7131
	YFTC 5256	Emory R., Morgan Co., TN	36 04 11	84 39 44	UT 91.7117
	YFTC 5276	French Broad, Sevier Co., TN	35 55 57	83 33 47	UT 91.7193
	YFTC 5640	Holston R., Jefferson Co., TN	36 06 02	83 4009	UT 91.7033
	YFTC 5641	Holston R., Jefferson Co., TN	36 06 02	83 4009	UT 91.7033
	YFTC 5668	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7043
	YFTC 5669	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7043
	YFTC 5670	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7043
	YFTC 5672	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7043
	YFTC 5673	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7043
	YFTC 6489	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7245
	YFTC 6490	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7245
	YFTC 6492	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7245
	YFTC 6493	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7245
	YFTC 6494	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7245
	YFTC 6503	Buck Cr., Pulaski Co., KY	37 09 07	84 26 17	UT 91.7297
	YFTC 6519	Sinking Cr., Laurel Co., KY	37 05 23	84 12 12	UT 91.7235
	YFTC 6520	Sinking Cr., Laurel Co., KY	37 05 23	84 12 12	UT 91.7235

Table C.1. Continued

Species	Catalogue	Locality	Lat(N)	Long(W)	Voucher
	YFTC 6521	Sinking Cr., Laurel Co., KY	37 05 23	84 12 12	UT 91.7235
	YFTC 6522	Sinking Cr., Laurel Co., KY	37 05 23	84 12 12	UT 91.7235
	YFTC 6526	Sinking Cr., Laurel Co., KY	37 05 23	84 12 12	UT 91.7235
	YFTC 6671	Clinch R., Hancock Co., TN	36 28 31	83 17 25	UT 91.7458
	YFTC 6672	Clinch R., Hancock Co., TN	36 28 31	83 17 25	UT 91.7458
	YFTC 6685	North Fk. Holston R., Hawkins Co., TN	36 31 58	82 36 31	UT 91.7443
	YFTC 6686	North Fk. Holston R., Hawkins Co., TN	36 31 58	82 36 31	UT 91.7443
	YFTC 6763	Holston R., Hawkins Co., TN	36 28 08	82 59 50	UT 91.7452
	YFTC 6764	Holston R., Hawkins Co., TN	36 28 08	82 59 50	UT 91.7452
	YFTC 7016	Rockcastle R., Laurel/Rockcastle Co., KY	37 16 46	84 12 35	UT 91.7519
	YFTC 7017	Rockcastle R., Laurel/Rockcastle Co., KY	37 16 46	84 12 35	UT 91.7519
	YFTC 7018	Rockcastle R., Laurel/Rockcastle Co., KY	37 16 46	84 12 35	UT 91.7519
	YFTC 7063	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7534
	YFTC 7064	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7534
	YFTC 7065	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7534
	YFTC 7066	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7534
	YFTC 7081	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7534
	YFTC 7082	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7534
	YFTC 8365	Red Bird R., Manchester Co., KY	37 11 28	83 35 27	YPM 16199
	YFTC 8416	South Fk. Kentucky R., Clay Co., KY	37 16 27	83 38 49	YPM 15807
	YFTC 9412	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16079
	YFTC 9413	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16079
	YFTC 9769	Elk R., Clay/Braxton Co. line, WV	38 35 11	80 56 07	YPM 20320
	YFTC 9909	Elk R., Clay/Braxton Co. line, WV	38 35 11	80 56 07	YPM 20320
	YFTC 9954	Elk R., Kanawha Co., WV	38 29 22	81 21 02	YPM 17090
	YFTC 9969	Elk R., Kanawha Co., WV	38 29 22	81 21 02	YPM 17090
	YFTC 9981	Licking R., Bath/Rowan Co. line, KY	38 10 33	83 37 06	YPM 17581
	YFTC 9986	Licking R., Bath/Rowan Co. line, KY	38 10 33	83 37 06	YPM 17581
	YFTC 11145	Kokosing R., Knox Co., OH	40 22 56	82 13 36	YPM 17296
	YFTC 11146	Kokosing R., Knox Co., OH	40 22 56	82 13 36	YPM 17296
	YFTC 11300	French Cr., Crawford Co., PA	41 46 17	80 06 34	YPM 17341
	YFTC 11324	Paint Cr., Ross Co., OH	39 15 48	83 10 01	YPM 17325
	YFTC 11325	Paint Cr., Ross Co., OH	39 15 48	83 10 01	YPM 17325
N. chlorobranchius	YFTC 2120	Burningtown Cr., Macon Co., NC	35 15 23	83 28 20	NCSM29472
	YFTC 2121	Burningtown Cr., Macon Co., NC	35 15 23	83 28 20	NCSM29472
	YFTC 3094	Little Pigeon R., Sevier Co., TN	35 44 21	83 24 59	UT 91.6577
	YFTC 5967	North Fk. Mills R., Henderson Co., NC	35 24 22	82 38 39	UT 91.7220
	YFTC 5968	North Fk. Mills R., Henderson Co., NC	35 24 22	82 38 39	UT 91.7220
	YFTC 5969	North Fk. Mills R., Henderson Co., NC	35 24 22	82 38 39	UT 91.7220
	YFTC 5970	North Fk. Mills R., Henderson Co., NC	35 24 22	82 38 39	UT 91.7220
	YFTC 5971	North Fk. Mills R., Henderson Co., NC	35 24 22	82 38 39	UT 91.7220
	YFTC 6802	North Indian Cr., Unicoi Co., TN	36 08 36	82 25 42	UT 91.7414
	YFTC 6803	North Indian Cr., Unicoi Co., TN	36 08 36	82 25 42	UT 91.7414
	YFTC 6804	North Indian Cr., Unicoi Co., TN	36 08 36	82 25 42	UT 91.7414
	YFTC 6805	North Indian Cr., Unicoi Co., TN	36 08 36	82 25 42	UT 91.7414
	YFTC 6806	North Indian Cr., Unicoi Co., TN	36 08 36	82 25 42	UT 91.7414
N. chuckwachatte	YFTC 5610	Cane Cr., Cleburne Co., AL	33 43 31	85 29 27	UT 91.6971
N. denoncourti	YFTC 2585	Clinch R., Claiborne Co., TN	36 25 48	83 23 49	UT 91.6530
	YFTC 4108	Sequatchie R., Sequatchie Co., TN	35 04 45	85 35 35	UT 91.6861
N. douglasi	YFTC 2116	Sipsey Fk., Winston Co., AL	34 17 07	87 23 57	INHS 55758
	YFTC 5540	Borden Cr., Lawrence Co., AL	34 19 46	87 22 37	UT 91.6963
N. etowahae	YFTC 2272	Etowah R., Lumpkin Co., GA	34 32 06	84 03 48	n.v.
	YFTC 4089	Shoal Cr., Dawson Co., GA	34 25 57	84 07 45	UT 91.1936
N. jordani	YFTC 9658	Talona Cr., Pickins Co., GA	34 31 36	84 30 34	YPM 16612
N. juliae	YFTC 2957	Buffalo R., Searcy Co., AR	35 59 08	92 44 41	UT 91.6639
	YFTC 2958	Buffalo R., Searcy Co., AR	35 59 08	92 44 41	UT 91.6639

Table C.1. Continued

Species	Catalogue	Locality	Lat(N)	Long(W)	Voucher
N. maculatus	YFTC 446	Green R., Green Co., KY	37 15 13	85 30 10	INHS 41700
	YFTC 9938	Elk R., Clay Co., WV	38 31 58	81 01 50	YPM 16821
	YFTC 9939	Elk R., Clay Co., WV	38 31 58	81 01 50	YPM 16821
	YFTC 11278	French Cr., Crawford Co., PA	41 46 17	80 06 34	YPM 17342
N. microlepidus	YFTC 2249	Harpeth R., Cheatham Co., TN	36 07 23	87 05 57	n.v.
	YFTC 5487	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7010
	YFTC 9381	Little R., Trigg Co., KY	36 46 41	87 43 16	YPM 16061
	YFTC 9410	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16080
N. moorei	YFTC 2884	Middle Fk. Little Red R., VanBuren Co., AR	35 31 20	92 26 26	UT 91.6658
	YFTC 2896	Middle Fk. Little Red R., VanBuren Co., AR	35 39 11	92 18 56	UT 91.6630
N. rubrus	YFTC 4912	Bayou Pierre, Copiah Co., MS	31 52 12	90 29 52	n.v.
	YFTC 4913	Bayou Pierre, Copiah Co., MS	32 00 10	90 41 22	n.v.
N. rufilineatus	YFTC 769	Grinders Cr., Lewis Co., TN	35 27 44	87 32 10	INHS 63880
	YFTC 770	Grinders Cr., Lewis Co., TN	35 27 44	87 32 10	INHS 63880
	YFTC 771	Grinders Cr., Lewis Co., TN	35 27 44	87 32 10	INHS 63880
	YFTC 772	Grinders Cr., Lewis Co., TN	35 27 44	87 32 10	INHS 63880
	YFTC 2370	Conasauga Cr., McMinn Co., TN	35 20 47	84 27 08	UT 91.6413
	YFTC 2371	Conasauga Cr., McMinn Co., TN	35 20 47	84 27 08	UT 91.6413
	YFTC 2372	Conasauga Cr., McMinn Co., TN	35 20 47	84 27 08	UT 91.6413
	YFTC 2374	Conasauga Cr., McMinn Co., TN	35 20 47	84 27 08	UT 91.6413
	YFTC 2375	Conasauga Cr., McMinn Co., TN	35 20 47	84 27 08	UT 91.6413
	YFTC 2491	Horse Cr., Hardin Co., TN	35 10 50	88 12 35	UT 91.6413
	YFTC 2492	Horse Cr., Hardin Co., TN	35 10 50	88 12 35	UT 91.6413
	YFTC 2493	Horse Cr., Hardin Co., TN	35 10 50	88 12 35	UT 91.6413
	YFTC 2494	Horse Cr., Hardin Co., TN	35 10 50	88 12 35	UT 91.6413
	YFTC 2505	Whites Cr., Cumberland Co., TN	35 48 49	84 51 40	UT 91.6505
	YFTC 2564	Duck R., Bedford Co., TN	35 33 15	86 34 59	UT 91.6609
	YFTC 2565	Duck R., Bedford Co., TN	35 33 15	86 34 59	UT 91.6609
	YFTC 2566	Duck R., Bedford Co., TN	35 33 15	86 34 59	UT 91.6609
	YFTC 2580	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.6528
	YFTC 2581	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.6528
	YFTC 2586	Clinch R., Claiborne Co., TN	36 25 48	83 23 49	UT 91.6531
	YFTC 3258	Holston R., Grainger Co., TN	36 00 26	83 49 36	UT 91.6748
	YFTC 3259	Holston R., Grainger Co., TN	36 00 26	83 49 36	UT 91.6748
	YFTC 3260	Holston R., Grainger Co., TN	36 00 26	83 49 36	UT 91.6748
	YFTC 4067	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.6771
	YFTC 4068	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.6771
	YFTC 4069	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.6771
	YFTC 4102	Sequatchie R., Sequatchie Co., TN	35 05 12	85 35 36	UT 91.6862
	YFTC 4104	Sequatchie R., Sequatchie Co., TN	35 05 12	85 35 36	UT 91.6862
	YFTC 4886	Rock Cr., Morgan Co., TN	36 08 01	84 37 25	UT 91.6872
	YFTC 4887	Rock Cr., Morgan Co., TN	36 08 01	84 37 25	UT 91.6872
	YFTC 4910	Rock Cr., Morgan Co., TN	36 08 01	84 37 25	UT 91.6875
	YFTC 4915	Emory R., Morgan Co., TN	36 04 08	84 39 44	UT 91.7141
	YFTC 4916	Emory R., Morgan Co., TN	36 04 08	84 39 44	UT 91.7141
	YFTC 4917	Emory R., Morgan Co., TN	36 04 08	84 39 44	UT 91.7141
	YFTC 4919	Emory R., Morgan Co., TN	36 04 08	84 39 44	UT 91.7141
	YFTC 5218	Helton Cr., DeKalb Co., TN	36 03 49	85 56 51	UT 91.7141
	YFTC 5219	Helton Cr., DeKalb Co., TN	36 03 49	85 56 51	UT 91.7141
	YFTC 5258	Emory R., Morgan Co., TN	36 04 08	84 39 44	n.v.
	YFTC 5259	Emory R., Morgan Co., TN	36 04 08	84 39 44	n.v.
	YFTC 5282	English Cr., Cocke Co., TN	35 55 24	83 13 04	UT 91.7182
	YFTC 5291	French Broad R., Knox Co., TN	35 57 06	83 41 58	UT 91.7209
	YFTC 5292	French Broad R., Knox Co., TN	35 57 06	83 41 58	UT 91.7209
	YFTC 5297	Cove Cr., Campbell Co., TN	36 24 51	84 18 05	UT 91.7173
	YFTC 5298	Cove Cr., Campbell Co., TN	36 24 51	84 18 05	UT 91.7173
	YFTC 5304	Cove Cr., Campbell Co., TN	36 24 51	84 18 05	UT 91.7189
	YFTC 5305	Old Town Cr., Claiborne Co., TN	36 31 07	83 43 07	UT 91.7189

Table C.1. Continued

Species	Catalogue	Locality	Lat(N)	Long(W)	Voucher
	YFTC 5477	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7011
	YFTC 5478	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7011
	YFTC 5479	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7011
	YFTC 5480	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7011
	YFTC 5481	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7011
	YFTC 5482	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7011
	YFTC 5484	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7011
	YFTC 5496	South Fk. Harpeth R., Williamson Co., TN	35 58 30	87 03 43	UT 91.7002
	YFTC 5497	South Fk. Harpeth R., Williamson Co., TN	35 58 30	87 03 43	UT 91.7002
	YFTC 5498	South Fk. Harpeth R., Williamson Co., TN	35 58 30	87 03 43	UT 91.7002
	YFTC 5499	South Fk. Harpeth R., Williamson Co., TN	35 58 30	87 03 43	UT 91.7002
	YFTC 5500	South Fk. Harpeth R., Williamson Co., TN	35 58 30	87 03 43	UT 91.7002
	YFTC 5503	South Fk. Harpeth R., Williamson Co., TN	35 58 30	87 03 43	UT 91.7002
	YFTC 5631	Ocoee R., Polk Co., TN	35 11 09	84 40 31	UT 91.7183
	YFTC 5632	Ocoee R., Polk Co., TN	35 11 09	84 40 31	UT 91.7183
	YFTC 5645	Holston R., Jefferson Co., TN	36 06 05	83 40 09	UT 91.7034
	YFTC 5646	Holston R., Jefferson Co., TN	36 06 05	83 40 09	UT 91.7034
	YFTC 5652	Hiwassee R., Polk Co., TN	35 14 23	84 33 46	UT 91.7039
	YFTC 5653	Hiwassee R., Polk Co., TN	35 14 23	84 33 46	UT 91.7039
	YFTC 5654	Hiwassee R., Polk Co., TN	35 14 23	84 33 46	UT 91.7039
	YFTC 5667	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7044
	YFTC 5747	Elk R., Moore/Franklin Co. line, TN	35 09 49	86 19 06	UT 91.7147
	YFTC 5748	Mulepen Cr., Lincoln Co., TN	35 03 09	86 25 12	UT 91.7096
	YFTC 5749	Mulepen Cr., Lincoln Co., TN	35 03 09	86 25 12	UT 91.7096
	YFTC 5771	Blackwater Cr., Hancock Co., TN	36 35 30	83 04 12	UT 91.7178
	YFTC 5774	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7195
	YFTC 5778	Smith Fk., Smith Co., TN	36 07 31	85 52 13	UT 91.7138
	YFTC 5779	Smith Fk., Smith Co., TN	36 07 31	85 52 13	UT 91.7138
	YFTC 5780	Smith Fk., Smith Co., TN	36 07 31	85 52 13	UT 91.7138
	YFTC 5781	Smith Fk., Smith Co., TN	36 07 31	85 52 13	UT 91.7138
	YFTC 5782	Smith Fk., Smith Co., TN	36 07 31	85 52 13	UT 91.7138
	YFTC 5783	Smith Fk., Smith Co., TN	36 07 31	85 52 13	UT 91.7138
	YFTC 5788	Smith Fk., Smith Co., TN	36 07 31	85 52 13	UT 91.7138
	YFTC 5800	Smith Fk., Smith Co., TN	36 07 31	85 52 13	UT 91.7138
	YFTC 5801	Smith Fk., Smith Co., TN	36 07 31	85 52 13	UT 91.7138
	YFTC 6469	Fishing Cr., Lincoln/Casey Co. line, KY	37 15 48	84 43 11	UT 91.7311
	YFTC 6470	Fishing Cr., Lincoln/Casey Co. line, KY	37 15 48	84 43 11	UT 91.7311
	YFTC 6471	Fishing Cr., Lincoln/Casey Co. line, KY	37 15 48	84 43 11	UT 91.7311
	YFTC 6472	Fishing Cr., Lincoln/Casey Co. line, KY	37 15 48	84 43 11	UT 91.7311
	YFTC 6473	Fishing Cr., Lincoln/Casey Co. line, KY	37 15 48	84 43 11	UT 91.7311
	YFTC 6474	Fishing Cr., Lincoln/Casey Co. line, KY	37 15 48	84 43 11	UT 91.7311
	YFTC 6476	Fishing Cr., Lincoln/Casey Co. line, KY	37 15 48	84 43 11	UT 91.7311
	YFTC 6477	Fishing Cr., Lincoln/Casey Co. line, KY	37 15 48	84 43 11	UT 91.7311
	YFTC 6478	Fishing Cr., Lincoln/Casey Co. line, KY	37 15 48	84 43 11	UT 91.7311
	YFTC 6488	Pitman Cr., Pulaski Co., KY	37 12 36	84 35 53	UT 91.7293
	YFTC 6594	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6595	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6596	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6597	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6598	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6599	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6600	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6601	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6602	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6603	Duck R., Coffee Co., TN	35 29 07	86 07 18	UT 91.7372
	YFTC 6641	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7272
	YFTC 6642	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7272
	YFTC 6644	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7272

Table C.1. Continued

Species	Catalogue	Locality	Lat(N)	Long(W)	Voucher
	YFTC 6646	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7272
	YFTC 6647	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7272
	YFTC 6649	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7272
	YFTC 6679	Powell R., Claiborne Co., TN	36 32 26	83 37 53	UT 91.7448
	YFTC 6687	North Fk. Holston R., Hawkins Co., TN	36 31 58	82 36 31	UT 91.7444
	YFTC 6697	Lynn Cr., Giles Co., TN	35 19 51	87 01 54	UT 91.7471
	YFTC 6698	Lynn Cr., Giles Co., TN	35 19 51	87 01 54	UT 91.7471
	YFTC 6699	Lynn Cr., Giles Co., TN	35 19 51	87 01 54	UT 91.7471
	YFTC 6765	Holston R., Hawkins Co., TN	36 28 09	82 50 59	UT 91.7453
	YFTC 6766	Holston R., Hawkins Co., TN	36 28 09	82 50 59	UT 91.7453
	YFTC 6807	West Fk. Shoal Cr., Giles Co., TN	35 04 40	87 09 06	UT 91.7460
	YFTC 6808	West Fk. Shoal Cr., Giles Co., TN	35 04 40	87 09 06	UT 91.7460
	YFTC 6831	Stewart Cr., Lincoln Co., TN	35 07 03	86 32 15	UT 91.7467
	YFTC 6832	Stewart Cr., Lincoln Co., TN	35 07 03	86 32 15	UT 91.7467
	YFTC 6834	Stewart Cr., Lincoln Co., TN	35 07 03	86 32 15	UT 91.7467
	YFTC 6835	Stewart Cr., Lincoln Co., TN	35 07 03	86 32 15	UT 91.7467
	YFTC 6836	Stewart Cr., Lincoln Co., TN	35 07 03	86 32 15	UT 91.7467
	YFTC 6858	Flat Cr., Knox Co., TN	36 06 39	83 43 49	UT 91.7465
	YFTC 6859	Flat Cr., Knox Co., TN	36 06 39	83 43 49	UT 91.7465
	YFTC 6882	Rock Cr., Colbert Co., AL	34 37 54	88 05 28	UT 91.7420
	YFTC 6883	Rock Cr., Colbert Co., AL	34 37 54	88 05 28	UT 91.7420
	YFTC 6884	Rock Cr., Colbert Co., AL	34 37 54	88 05 28	UT 91.7420
	YFTC 6958	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7511
	YFTC 6959	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7511
	YFTC 6960	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7511
	YFTC 6961	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7511
	YFTC 6962	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7511
	YFTC 6963	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7511
	YFTC 6964	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7511
	YFTC 6966	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7511
	YFTC 7121	French Broad, Cocke Co., TN	35 56 24	82 55 47	YPM 15912
	YFTC 7123	French Broad, Cocke Co., TN	35 56 24	82 55 47	YPM 15912
	YFTC 7124	French Broad, Cocke Co., TN	35 56 24	82 55 47	YPM 15912
	YFTC 7125	French Broad, Cocke Co., TN	35 56 24	82 55 47	YPM 15912
	YFTC 7210	West Fk. Obey R., Overton Co., TN	36 17 29	85 11 51	YPM 18508
	YFTC 7212	West Fk. Obey R., Overton Co., TN	36 17 29	85 11 51	YPM 18508
	YFTC 7314	Hinds Cr., Knox/Union Co., TN	36 13 32	83 54 16	YPM 16180
	YFTC 7383	East Fk. Shoal Cr., Giles Co., TN	35 02 28	87 05 02	YPM 18422
	YFTC 7384	East Fk. Shoal Cr., Giles Co., TN	35 02 28	87 05 02	YPM 18422
	YFTC 7385	East Fk. Shoal Cr., Giles Co., TN	35 02 28	87 05 02	YPM 18422
	YFTC 7463	Cedar Cr., Franklin Co., AL	34 30 05	87 50 02	YPM 16346
	YFTC 7464	Cedar Cr., Franklin Co., AL	34 30 05	87 50 02	YPM 16346
	YFTC 7465	Cedar Cr., Franklin Co., AL	34 30 05	87 50 02	YPM 16346
	YFTC 7466	Cedar Cr., Franklin Co., AL	34 30 05	87 50 02	YPM 16346
	YFTC 7467	Cedar Cr., Franklin Co., AL	34 30 05	87 50 02	YPM 16346
	YFTC 7563	Little Bear Cr., Franklin Co., AL	34 22 59	87 50 10	YPM 16228
	YFTC 7592	Second Cr., Lauderdale Co., AL	34 57 21	88 01 28	YPM 16322
	YFTC 7593	Second Cr., Lauderdale Co., AL	34 57 21	88 01 28	YPM 16322
	YFTC 7615	Indian Cr., Hardin Co., TN	35 16 36	88 01 18	YPM 16598
	YFTC 7627	Indian Cr., Hardin Co., TN	35 16 36	88 01 18	YPM 16598
	YFTC 7628	Indian Cr., Hardin Co., TN	35 16 36	88 01 18	YPM 16598
	YFTC 7654	Hardin Cr., Wayne Co., TN	35 18 26	87 57 11	YPM 16116
	YFTC 7655	Hardin Cr., Wayne Co., TN	35 18 26	87 57 11	YPM 16116
	YFTC 7656	Hardin Cr., Wayne Co., TN	35 18 26	87 57 11	YPM 16116
	YFTC 8219	McWilliams Cr., Sequatchie Co., TN	35 24 07	85 20 21	YPM 15696
	YFTC 8220	McWilliams Cr., Sequatchie Co., TN	35 24 07	85 20 21	YPM 15696
	YFTC 8226	McWilliams Cr., Sequatchie Co., TN	35 24 07	85 20 21	YPM 15696
	YFTC 8287	Mill Cr., Clay Co., TN	36 29 39	85 33 02	YPM 15615

Table C.1. Continued

Species	Catalogue	Locality	Lat(N)	Long(W)	Voucher
	YFTC 8289	Mill Cr., Clay Co., TN	36 29 39	85 33 02	YPM 15615
	YFTC 8308	Helton Cr., Smith Co., TN	36 03 48	85 56 53	YPM 15682
	YFTC 9358	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9359	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9360	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9361	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9362	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9363	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9364	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9365	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9366	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9367	West Fk. Clarks R., Calloway Co., KY	36 44 27	88 27 41	YPM 16225
	YFTC 9397	Yellow Cr., Houston Co., TN	36 17 50	87 32 58	YPM 17534
	YFTC 9398	Yellow Cr., Houston Co., TN	36 17 50	87 32 58	YPM 17534
	YFTC 9399	Yellow Cr., Houston Co., TN	36 17 50	87 32 58	YPM 17534
	YFTC 9408	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16081
	YFTC 9409	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16081
	YFTC 9418	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16081
	YFTC 9436	Blackburn Fk., Jackson Co., TN	36 19 42	85 33 50	YPM 16138
	YFTC 9437	Blackburn Fk., Jackson Co., TN	36 19 42	85 33 50	YPM 16138
	YFTC 9438	Blackburn Fk., Jackson Co., TN	36 19 42	85 33 50	YPM 16138
	YFTC 9442	Wolf R., Pickett Co., TN	36 34 11	85 05 54	YPM 16285
	YFTC 9443	Wolf R., Pickett Co., TN	36 34 11	85 05 54	YPM 16285
	YFTC 9444	Wolf R., Pickett Co., TN	36 34 11	85 05 54	YPM 16285
	YFTC 9452	Otter Cr., Wayne Co., KY	36 42 20	84 56 37	YPM 16195
	YFTC 9453	Otter Cr., Wayne Co., KY	36 42 20	84 56 37	YPM 16195
N. sanguifluus	YFTC 940	Rockcastle R., Rockcastle Co., KY	37 17 33	84 13 14	n.v.
	YFTC 2444	Scott Cr., Warren Co., TN	35 34 17	84 42 43	UT 91.6706
	YFTC 6505	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7298
	YFTC 7068	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7535
N. tippecanoe	YFTC 6424	Green R., Green Co., KY	37 15 59	85 34 52	UT 91.7305
	YFTC 7084	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7536
	YFTC 9933	Elk R., Clay/Braxton Co. line, WV	38 35 11	80 56 07	YPM 20321
N. vulneratus	YFTC 460	Daddys Cr., Cumberland Co., TN	36 03 32	84 47 32	n.v.
	YFTC 2203	Taccoa R., Fannin Co., GA	34 45 51	84 14 56	TU 197315
	YFTC 2204	Oconoluftee R., Jackson Co., NC	35 28 07	83 19 28	TU 197316
	YFTC 2588	Clinch R., Claiborne Co., TN	36 25 48	83 23 49	UT 91.6532
	YFTC 5776	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7196
	YFTC 6965	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7512
N. wapiti	YFTC 4097	Elk R., Giles/Lincoln Co., TN	35 04 56	86 49 60	UT 91.686
	YFTC 4098	Elk R., Giles/Lincoln Co., TN	35 04 56	86 49 60	UT 91.686

Table C.2. Optimal models of molecular evolution selected using AIC by data partition for each gene.

Gene Partition	Model
cyt*b*	
1st codon	K81uf + I + G
2nd codon	GTR + I
3rd codon	GTR + I + G
MLL	
1st codon	TrN + I
2nd codon	HKY + G
3rd codon	F81
S7	
all codons	TIM + I + G
RAG1	
1st codon	TVM + G
2nd codon	GTR
3rd codon	HKY

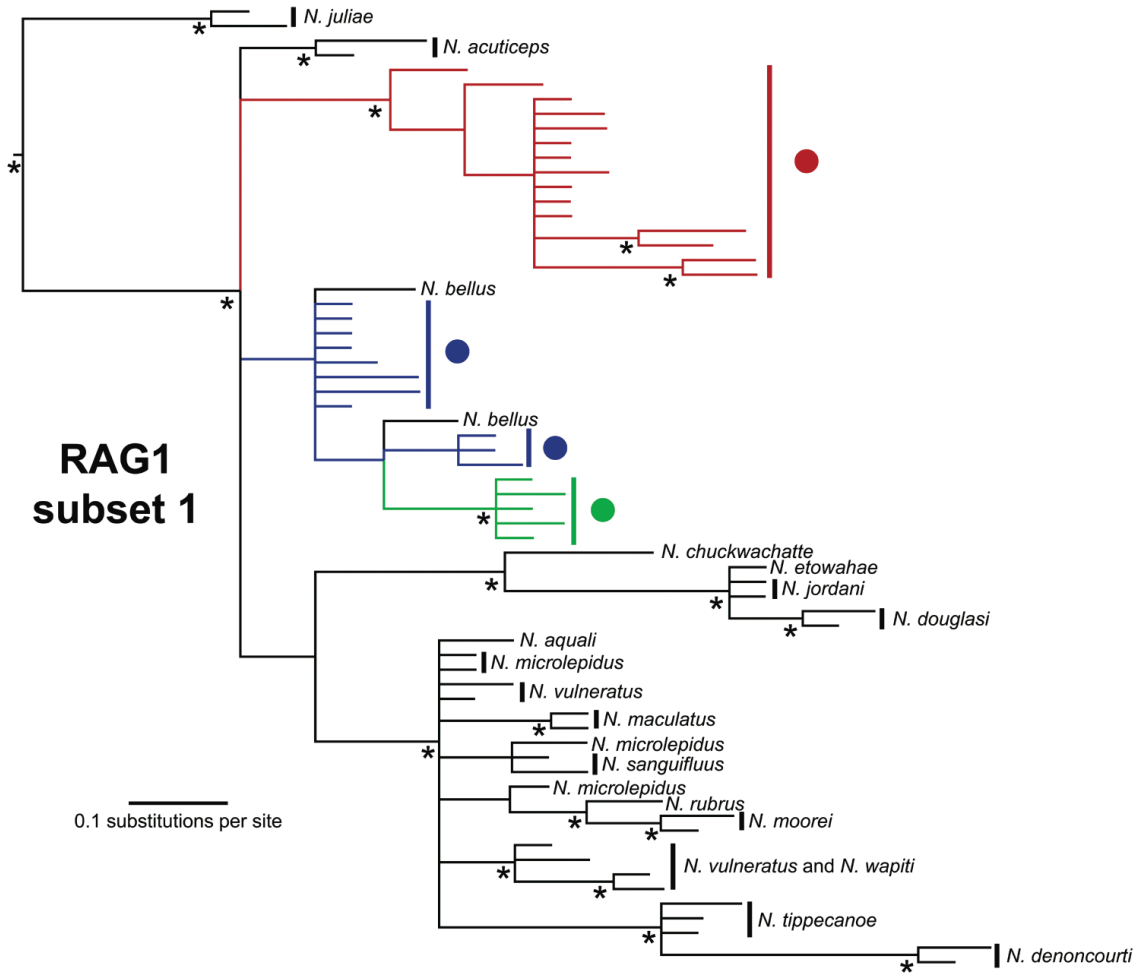

Fig. C.1. Phylogeny resulting from Bayesian analysis of a RAG1 exon dataset containing half the number of *N. rufilineatus* alleles as the complete dataset. All nodes supported with a Bayesian posterior probability of > .94 are indicated with asterisks. Blue dots indicate alleles isolated from *N. camurus* individuals, green dots indicate alleles isolated from *N. chlorobranchius* individuals, and red dots indicate alleles isolated from *N. rufilineatus* individuals.

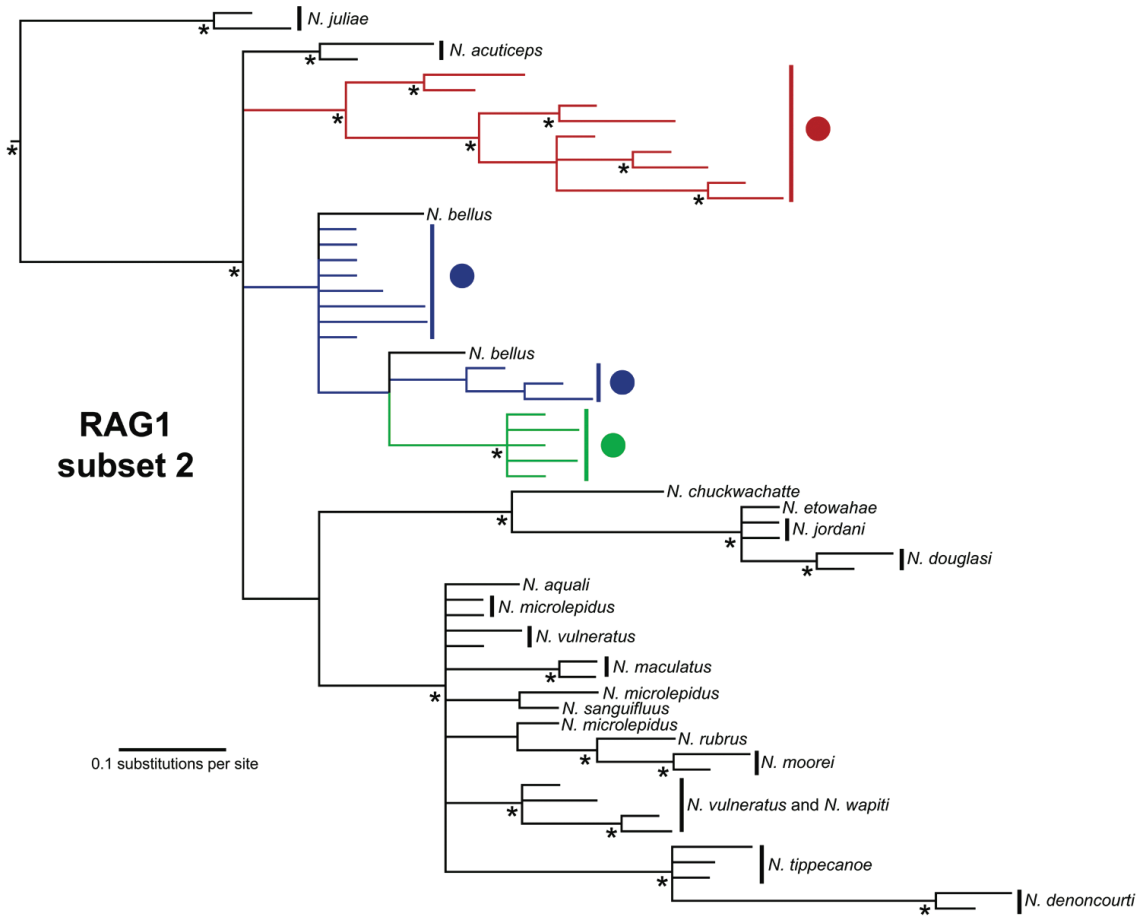

Fig. C.2. Phylogeny resulting from Bayesian analysis of a RAG1 exon dataset containing fewer *N. rufilineatus* alleles than *N. camurus* alleles. All nodes supported with a Bayesian posterior probability of > .94 are indicated with asterisks. Blue dots indicate alleles isolated from *N. camurus* individuals, green dots indicate alleles isolated from *N. chlorobranchius* individuals, and red dots indicate alleles isolated from *N. rufilineatus* individuals.

APPENDIX D
Supplemental material for Chapter 5

Table D.1. Specimens used in genetic analyses. Catalogue number (YFTC=Yale Fish Tissue Collection, BPO1=from Dr. B. Porter, Duquesne Univ.), specific locality, latitude, longitude, and voucher (INHS=Illinois Natural History Survey, UT=University of Tennessee Research Collection of Fishes, YPM=Yale Peabody Museum of Natural History).

Species	Catalogue #	Locality	Lat.(N)	Long.(W)	Voucher
Perca flavescens	YFTC 261	Lake Andrusia, Beltrami Co., MN	47 27 23	94 39 13	NHS 39508
Percina roanoka	YFTC 76	Blackwater R., Franklin Co., VA	37 03 14	79 52 56	INHS 64359
Crystallaria asprella	YFTC 686	Cahaba R., Cumberland Co., IL	32 56 52	87 08 26	n.v.
Etheostoma blennioides	YFTC 756	West Fk. Pond R., Christian Co., KY	37 01 52	87 24 21	INHS 32700
Nothonotus acuticeps	YFTC 5659	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7042
N. aquali	YFTC 68	Buffalo R., Lewis Co., TN	35 27 44	87 32 10	n.v.
	YFTC 2562	Duck R., Bedford Co., TN	35 33 12	86 34 57	UT 91.6607
N. camurus	YFTC 1203	Middle Fk. Vermillion R., Vermillion Co., IL	40 14 06	87 46 18	n.v.
N. jordani	YFTC 2366	Conasauga R., Polk Co., TN	35 00 09	84 46 44	UT 91.6541
N. juliae	YFTC 2957	Buffalo R., Searcy Co., AR	35 59 08	92 44 41	UT 91.6639
N. maculatus	BPO1	French Cr., Crawford Co., PA	-	-	n.v.
	YFTC 446	Green R., Green Co., KY	37 15 13	85 30 10	INHS 41700
	YFTC 9938	Elk R., Clay Co., WV	38 31 58	81 01 50	YPM 16821
	YFTC 9939	Elk R., Clay Co., WV	38 31 58	81 01 50	YPM 16821
	YFTC 9940	Elk R., Clay Co., WV	38 31 58	81 01 50	YPM 16821
	YFTC 9941	Elk R., Clay Co., WV	38 31 58	81 01 50	YPM 16821
	YFTC 10056	Green R., Green Co., KY	37 15 13	85 30 09	YPM 17648
	YFTC 10056	Green R., Green Co., KY	37 15 13	85 30 09	YPM 17648
	YFTC 11158	Kokosing R., Knox Co., OH	40 22 55	82 13 37	YPM 17297
	YFTC 11159	Kokosing R., Knox Co., OH	40 22 55	82 13 37	YPM 17297
	YFTC 11278	French Cr., Crawford Co., PA	41 46 17	80 06 34	YPM 17342
	YFTC 11279	French Cr., Crawford Co., PA	41 46 17	80 06 34	YPM 17342
	YFTC 11281	French Cr., Crawford Co., PA	41 46 17	80 06 34	YPM 17342
N. microlepidus	YFTC 2249	Harpeth R., Cheatham Co., TN	36 07 23	87 05 57	n.v.
	YFTC 5487	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7010
	YFTC 5488	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7010
	YFTC 5489	East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	UT 91.7010
	YFTC 7174	East Fk. Stones R., Rutherford Co., TN	35 54 48	86 19 49	UT 91.7623
	YFTC 7176	East Fk. Stones R., Rutherford Co., TN	35 54 48	86 19 49	UT 91.7623
	YFTC 7177	East Fk. Stones R., Rutherford Co., TN	35 54 48	86 19 49	UT 91.7623
	YFTC 7178	East Fk. Stones R., Rutherford Co., TN	35 54 48	86 19 49	UT 91.7623
	YFTC 9381	Little R., Trigg Co., KY	36 46 41	87 43 16	YPM 16061
	YFTC 9382	Little R., Trigg Co., KY	36 46 41	87 43 16	YPM 16061
	YFTC 9383	Little R., Trigg Co., KY	36 46 41	87 43 16	YPM 16061
	YFTC 9384	Little R., Trigg Co., KY	36 46 41	87 43 16	YPM 16061
	YFTC 9385	Little R., Trigg Co., KY	36 46 41	87 43 16	YPM 16061
	YFTC 9386	Little R., Trigg Co., KY	36 46 41	87 43 16	YPM 16061
	YFTC 9410	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16080
	YFTC 9411	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16080
	YFTC 9421	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16080
	YFTC 9422	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16080
	YFTC 9423	Red R., Robertson Co., TN	36 35 14	87 03 39	YPM 16080
N. moorei	YFTC 2896	Middle Fk. Little Red R., Van Buren Co., AR	35 39 11	92 18 56	UT 91.6630
	YFTC 2898	Middle Fk. Little Red R., Van Buren Co., AR	35 39 11	92 18 56	UT 91.6630
	YFTC 2884	South Fk. Little Red R., Van Buren Co., AR	35 31 20	92 26 26	UT 91.6658
	YFTC 9386	Beech Fk., Cleburne Co., AR	35 37 57	92 03 10	n.v.
N. rubrus	YFTC 4912	Bayou Pierre, Copiah Co., MS	31 52 12	90 29 52	n.v.
	YFTC 4913	Bayou Pierre, Copiah Co., MS	32 00 10	90 41 22	n.v.
	YFTC 4914	Foster's Cr., Copiah Co., MS	31 55 52	90 37 07	n.v.
N. rufilineatus	YFTC 2493	Horse Cr., Hardin Co., TN	35 10 50	88 12 35	UT 91.6413
N. sanguifluus	YFTC 940	Rockcastle R., Rockcastle Co., KY	37 17 33	84 13 14	n.v.
	YFTC 2444	Scott Cr., Warren Co., TN	35 34 17	84 42 43	UT 91.6706
	YFTC 2446	Scott Cr., Warren Co., TN	35 34 17	84 42 43	UT 91.6706
	YFTC 2464	Collins R., Warren Co., TN	35 44 46	85 41 55	UT 91.6563
	YFTC 2465	Collins R., Warren Co., TN	35 44 46	85 41 55	UT 91.6563

Table D.1. Continued.

Species	Catalogue #	Locality	Lat.(N)	Long.(W)	Voucher
	YFTC 3128	Cane Cr., VanBuren Co., TN.	35 46 57	85 24 16	UT 91.6632
	YFTC 3129	Cane Cr., VanBuren Co., TN.	35 46 57	85 24 16	UT 91.6632
	YFTC 3130	Cane Cr., VanBuren Co., TN.	35 46 57	85 24 16	UT 91.6632
	YFTC 3131	Cane Cr., VanBuren Co., TN.	35 46 57	85 24 16	UT 91.6632
	YFTC 3150	Caney Fk. R., Whites Co., TN.	35 48 44	85 22 26	UT 91.6594
	YFTC 3169	Cane Cr., VanBuren Co., TN.	35 46 57	85 24 16	UT 91.6596
	YFTC 3170	Cane Cr., VanBuren Co., TN.	35 46 57	85 24 16	UT 91.6596
	YFTC 6499	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7298
	YFTC 6500	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7298
	YFTC 6504	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7298
	YFTC 6505	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7298
	YFTC 6506	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7298
	YFTC 6507	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	UT 91.7298
	YFTC 6563	Scott Cr., Warren Co., TN	35 34 17	84 42 43	UT 91.7376
	YFTC 6564	Scott Cr., Warren Co., TN	35 34 17	84 42 43	UT 91.7376
	YFTC 7021	Rockcastle R., Laurel/Rockcastle Co., KY	37 17 16	84 12 23	UT 91.7520
	YFTC 7022	Rockcastle R., Laurel/Rockcastle Co., KY	37 17 16	84 12 23	UT 91.7520
	YFTC 7023	Rockcastle R., Laurel/Rockcastle Co., KY	37 17 16	84 12 23	UT 91.7520
	YFTC 7024	Rockcastle R., Laurel/Rockcastle Co., KY	37 17 16	84 12 23	UT 91.7520
	YFTC 7031	Rockcastle R., Laurel/Rockcastle Co., KY	37 17 16	84 12 23	UT 91.7520
	YFTC 7067	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7535
	YFTC 7068	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7535
	YFTC 7069	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7535
	YFTC 7070	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7535
	YFTC 7071	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7535
	YFTC 7072	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7535
	YFTC 7077	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7535
	YFTC 8243	Charles Cr., Warren Co., TN	35 42 59	85 46 01	YPM 15554
	YFTC 9467	Rockcastle R., Laurel Co., KY	37 14 26	84 14 21	YPM 16186
	YFTC 9468	Rockcastle R., Laurel Co., KY	37 14 26	84 14 21	YPM 16186
N. tippecanoe	YFTC 6424	Green R., Green Co., KY	37 15 59	85 34 52	UT 91.7305
N. vulneratus	YFTC 6424	Green R., Green Co., KY	37 15 59	85 34 52	UT 91.7305
	YFTC 7084	Big South Fk., Scott Co., TN	36 32 49	84 39 56	UT 91.7536
	YFTC 2591	Clinch R., Claiborne Co., TN	36 25 48	83 23 49	UT 91.6532
	YFTC 2588	Clinch R., Claiborne Co., TN	36 25 48	83 23 49	UT 91.6532
	YFTC 460	Daddys Cr., Cumberland Co., TN	36 03 32	84 47 32	n.v.
	YFTC 5677	Nolichucky R., Greene Co., TN	36 06 00	83 02 51	UT 91.7044
	YFTC 5776	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7196
	YFTC 5777	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7196
	YFTC 6965	Taccoa R., Fannin Co., GA	34 45 27	84 11 57	UT 91.7512
	YFTC 6638	Little R., Blount Co., TN	35 47 16	83 52 55	UT 91.7271
N. wapiti	YFTC 4097	Elk R., Giles/Lincoln Co., TN	35 04 56	86 49 60	UT 91.6860
	YFTC 4098	Elk R., Giles/Lincoln Co., TN	35 04 56	86 49 60	UT 91.6860
	YFTC 4099	Elk R., Giles/Lincoln Co., TN	35 04 56	86 49 60	UT 91.6860
	YFTC 4100	Elk R., Giles/Lincoln Co., TN	35 04 56	86 49 60	UT 91.6860

Table D.2. Specimens sampled for morphometric analysis. Catalogue number (CU=Cornell University Museum of Vertebrates, UT=University of Tennessee Research Collection of Fishes, YPM=Yale Peabody Museum of Natural History), tributary of Cumberland River sampled from, geographic locality, latitude, longitude, and number of individuals measured/number of specimens in lot.

Species	Catalogue #	Tributary	Locality	Lat.(N)	Long.(W)	Ind.
N. sanguifluus	UT 91.4860	Big South Fk.	Beech Fk., Campbell Co., TN	36 14 12	84 19 46	5/5
	UT 91.4649		Big South Fk., Scott Co., TN	36 28 34	84 40 08	1/1
	UT 91.440		Big South Fk., Scott Co., TN	36 28 34	84 40 08	5/5
	UT 91.7535		Big South Fk., Scott Co., TN	36 32 49	84 39 56	10/11
	UT 91.4297		Big South Fk., Scott Co., TN	36 32 49	84 39 56	5/5
	UT 91.668		Big South Fk., Scott Co., TN	36 32 49	84 39 56	7/7
	UT 91.443		Big South Fk., Scott Co., TN	36 32 49	84 39 56	12/12
	UT 91.453		Big South Fk., Scott Co., TN	36 32 49	84 39 56	40/54
	UT 91.4845		Cages Cr., Anderson Co., TN	36 12 07	84 19 50	2/2
	UT 91.1472		Clear Fk. R., Scott Co., TN	36 23 16	84 37 47	12/12
	UT 91.442		Clear Fk. R., Scott Co., TN	36 23 16	84 37 47	20/20
	UT 91.666		Little South Fk., McCreary Co., KY	36 45 29	84 40 20	3/4
	UT 91.4897		New R., Anderson Co., TN	36 12 04	84 19 47	4/4
	UT 91.4935		New R., Anderson Co., TN	36 12 39	84 19 19	8/8
	UT 91.4909		New R., Campbell Co., TN	36 16 08	84 20 40	10/10
	UT 91.2288		New R., Scott Co., TN	36 20 11	84 27 06	2/2
	UT 91.4966		New R., Scott Co., TN	36 23 02	84 33 11	10/10
	UT 91.5203		New R., Scott Co., TN	36 24 15	84 35 31	9/15
	UT 91.4953		Nicks Cr., Campbell Co., TN	36 16 06	84 20 12	2/2
	UT 91.403		No Business Cr., Scott Co., TN	36 35 10	84 40 24	1/1
	UT 91.437		North Whiteoak Cr., Scott Co., TN	36 27 21	84 40 18	7/7
	UT 91.5020		Paint Rock Cr., Scott Co., TN	36 23 34	84 27 26	4/4
	UT 91.4865		Phillips Cr., Scott Co., TN	36 23 11	84 32 58	1/2
	UT 91.3254		Smokey Cr., Scott Co., TN	36 16 38	84 22 29	1/1
	UT 91.4973		Smokey Cr., Scott Co., TN	36 16 38	84 22 29	14/14
	UT 91.7298	Buck Cr.	Buck Cr., Pulaski Co., KY	37 18 34	84 33 59	9/10
	UT 91.2522	Caney Fk.	Barren Fk., Warren Co., TN	35 40 04	85 49 12	2/2
	UT 91.2522		Barren Fk., Warren Co., TN	35 40 04	85 49 12	7/7
	UT 91.108		Barren Fk., Warren Co., TN	35 40 04	85 49 12	3/3
	UT 91.108		Barren Fk., Warren Co., TN	35 40 04	85 49 12	14/14
	CU 65364		Cane Cr., Spencer Co., TN	35 47 44	85 24 34	1/1
	UT 91.2753		Cane Cr., Van Buren Co., TN	35 44 35	85 23 37	4/4
	UT 91.2753		Cane Cr., Van Buren Co., TN	35 46 15	85 24 05	1/1
	UT 91.6596		Cane Cr., Van Buren Co., TN	35 46 57	85 24 16	2/2
	UT 91.3055		Charles Cr., Warren Co., TN	35 43 04	85 45 30	6/6
	UT 91.520		Charles Cr., Warren Co., TN	35 43 04	85 45 30	53/53
	UT 91.108		Collins R., Grundy Co., TN	35 30 10	85 39 50	1/1
	UT 91.6942		Collins R., Warren Co., TN	35 40 31	85 42 33	1/1
	CU 51609		Collins R., Warren Co., TN	35 40 31	85 42 34	69/109
	UT 91.644		Collins R., Warren Co., TN	35 42 31	85 43 50	26/26
	UT 91.6563		Collins R., Warren Co., TN	35 44 46	85 41 55	9/12
	UT 91.8		Mountain Cr., Warren Co., TN	35 46 56	85 47 27	3/3
	UT 91.2361		Rocky R., Van Buren Co., TN	35 42 04	85 34 40	1/1
	UT 91.6706		Scott Cr., Warren Co., TN	35 34 17	84 42 43	5/5
	UT 91.7376		Scott Cr., Warren Co., TN	35 34 17	84 42 43	7/7
	CU 50126	Roaring R.	Roaring R., Jackson Co., TN	36 21 04	85 32 45	1/1
	UT 91.268		Roaring R., Jackson Co., TN	36 21 47	85 36 50	2/2
	UT 91.6840	Rockcastle R.	Horse Lick Cr., Jackson Co., KY	37 25 48	84 08 27	1/1
	YPM 16186		Rockcastle R., Laurel Co., KY.	37 14 26	84 14 21	20/20
	UT 91.1226		Rockcastle R., Laurel/Pulaski Co., KY	37 08 43	84 17 37	1/1
	CU 47380		Rockcastle R., Laurel/Pulaski Co., KY	37 08 43	84 17 37	8/8
	UT 91.7520		Rockcastle R., Laurel/Pulaski Co., KY	37 17 16	84 12 23	15/15
	UT 91.186	Wolf R.	Wolf R., Pickett Co., TN	36 34 59	85 07 03	7/7
	CU 51350		Wolf R., Pickett Co., TN	36 34 59	85 07 03	8/8

Table D.2. Continued.

Species	Catalogue #	Tributary	Locality	Lat.(N)	Long.(W)	Ind.
N. microlepidus	UT 91.2387	Harpeth R.	Big Turnbull Cr., Cheatham Co., TN	36 05 44	87 08 28	8/9
	UT 91.711		Big Turnbull Cr., Cheatham Co., TN	36 05 44	87 08 28	14/14
	UT 91.3115		Harpeth R.	na	na	3/3
	UT 91.5239		Harpeth R., Cheatham Co., TN	36 08 10	87 05 59	3/8
	UT 91.1129		Harpeth R., Cheatham Co., TN	36 08 57	87 07 14	6/6
	UT 91.972		Harpeth R., Davidson Co., TN	36 03 15	86 56 54	1/1
	YPM 16061	Little R.	Little R., Trigg Co., KY	36 46 41	87 43 16	22/22
	UT 91.3739	Red R.	Red R., Robertson Co., TN	36 35 14	87 03 39	19/19
	YPM 16080		Red R., Robertson Co., TN	36 35 14	87 03 39	7/7
	UT 91.3030	Stones R.	East Fk. Stones R., Cannon Co., TN	35 49 41	86 04 37	15/15
	UT 91.7010		East Fk. Stones R., Rutherford Co., TN	35 52 57	86 16 22	3/3
	UT 91.7623		East Fk. Stones R., Rutherford Co., TN	35 54 48	86 19 49	5/5
	UT 91.2235		West Fk. Stones R., Rutherford Co., TN	35 56 28	86 27 48	2/2

Table D.3. Optimal models of molecular evolution selected using AIC by data partition for each gene.

Gene Partition	Model
cyt*b*	
1st codon	TrNef + I + G
2nd codon	F81
3rd codon	GTR + I + G
all codons	GTR + I + G
MLL	
1st codon	F81 + I
2nd codon	K80
3rd codon	F81
all codons	TrN
S7	
all codons	TIM + I + G
RAG1	
1st codon	HKY + G
2nd codon	HKY
3rd codon	HKY
all codons	TrN + G

VITA

Benjamin Paul Keck was born on 31 March 1977, in Louisville, Kentucky. He attended public school in Jefferson Co., Kentucky, through the 2nd grade and in Oldham Co., Kentucky, until he graduated from South Oldham High School in 1995. In 1999 he graduated from Centre College of Kentucky earning a Bachelor of Science degree with a major in biology and a minor in anthropology. He enrolled at the University of Tennessee, Knoxville, in 2000, working with Dave Etnier, and received the Master of Science degree with a major in Ecology and Evolutionary Biology in December 2003. For his Masters research he conducted an ichthyofaunal survey of the Hatchie River system in west Tennessee and north Mississippi. He continued in the Department of Ecology and Evolutionary Biology at the University of Tennessee for his Ph.D. research, working with Tom Near, and passed his defense in October 2008. He will begin a postdoctoral position in the Department of Ecology and Evolutionary Biology at Yale University in August 2009. He hopes to eventually find a professorship at college or university where he can integrate undergraduate education with a strong research program.